

Embryo-toxic effects of lead nitrate of the African catfish
***Clarias gariepinus* (Burchell, 1822)**

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I dedicate this thesis
To the memory of my Father,
To my mother,
To my lovely wife Sahar,
And
To my pretty kids, Zeyad and Shaza

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CHAPTER 1

General Introduction

1.1 Water Pollution in Egypt

Water pollution is one of the environmental and public health problems Egypt and the Middle East region are facing (Anwar, 2003). In Egypt, pollution is generally associated with heavy industrialisation and dense population and is one of the ecological problems of the river Nile system (Ali and Soltan, 1996). The river Nile is the principal freshwater resource for the country, meeting nearly all demands for drinking water, irrigation, and industry (Mohamed *et al.*, 1998). The pollution of the river Nile resulted from many sources, e.g. accidental spillage and deficiencies in the treatment of chemical wastes, discharge of industrial or sewerage effluents, domestic wastewater, the disposal of untreated sewage and gasoline from fishery boats (Handy, 1994; Ali and Soltan, 1996; Mohamed *et al.*, 1998). Furthermore, modern agricultural activities have introduced several polluting substances such as organic matter, chemical fertilizer, and insecticides into the river Nile and the drainage systems.

1.1.1 Heavy Metals

Heavy metals are the important source of pollution for aquatic habitats. In spite of their natural occurrence in the aquatic ecosystem, heavy metals represent a major problematic environmental issue of increasing concern (Gill *et al.*, 1990; Hunaiti and Soud, 2000) and their monitoring has received worldwide significant attention in the field and under laboratory conditions (Christensen, 1975; Gupta and Sastry, 1981; Adham *et al.*, 1999; Gilli *et al.*, 2000; Almeida *et al.*, 2001; Rashed, 2001b; Adham, 2002; Almeida *et al.*, 2002; Elumalai *et al.*, 2002; Strmac and Braunbeck, 2002; Long *et al.*, 2003; Pandey *et al.*, 2003; Barnhoorn and van Vuren, 2004; Das *et al.*, 2004a; Das *et al.*, 2004b). The presence of heavy metals in different kind of food constitutes serious health hazards, depending on their relative levels. Lead for example causes renal failure and liver damage (Emmerson, 1973). Some other metals cause poor reproductive capacity, hypertension and tumours and hepatic dysfunction (Mansour and Sidky, 2002). The level of heavy metals in the water and in the sediment of some parts of the river Nile is higher than the tolerance levels or limits set by the Egyptian General Authority for Standards and Quality Control (Anwar, 2003).

1.1.2 Lead

Lead (Pb) seems to lack biological functions and is very toxic for aquatic organisms even in small doses (Sastry and Gupta, 1980; Stouthart *et al.*, 1994; Ahmed, 1996; Lashein, 1996). It is one of the most dangerous pollutants in our environment which accumulates in the body due to its low rate of elimination (Harrison and Winchest, 1971). Lead enters aquatic systems from urban, mining and agricultural runoff, atmospheric precipitation, plating process, the use of phosphate fertilizers and gasoline containing lead that leaks from fishery boats and a variety of natural sources, including erosion and volcanic emissions (Pascoe and Matthey, 1977; Denny *et al.*, 1987; Handy, 1994). The anthropogenic release of Pb to the environment is the highest of all heavy metals (Pb > Ag > Mo > Sb > Zn > Cd > As > Cr > Co > Mn > Hg; Salomons and Förstner, 1984). About 3.3 million tones of lead were mined annually worldwide; so many countries have the problem of lead pollution (Mason, 1991; Hussein and Mekkawy, 2001).

Recent studies reported high lead contamination in Egypt in the range of 2000 mg/l in soil and 400 mg/l in river sediments (Elsokkary and Muller, 1990; Elsokkary *et al.*, 1995), and in food such as fish, spices and milk products at mean concentrations above the permissible limits proposed by FAO (Dogheim *et al.*, 1988; Abo-Gharbia and El-Sawi, 1999; Mansour and Sidky, 2002; Dogheim *et al.*, 2004) in contrast to the general trend for decreasing lead concentrations reported from European and North American countries via introduction of Pb free gasoline and recycling of Pb. Lead contamination is recognized as a major problem and the Egyptian State Ministry of Environment initiated new policies that include risk minimization and law enforcement (Anwar, 2003), calling for suitable monitoring systems. In Egypt, environmental risk assessment and environmental biomonitoring is scarce, but initiatives like this study are a first step for improvement.

1.2 Fish embryos as test organisms

The sensitivity of organisms to pollutants varies among species, population and life stage (Woltering, 1984). Therefore, it is important to choose the most suitable test organism. Fish have been the most popular choice as test organism because they are presumably the best-understood organisms in the aquatic environment and also due to their importance to man as a protein source (Kime *et al.*, 1996). As a result, aquatic

pollution influences human indirectly through the ingestion of fish due to bioaccumulation. It is therefore of great significance to evaluate pollution effects on fish for both environmental protection and socio-economic reasons (Lin and Hwang, 1998). Fish might prove a better media than water for detecting heavy metal contamination (Barak and Mason, 1990) because they concentrate large amounts of some metals from the water (Mansour and Sidky, 2002). Fish size can have a significant effect on the impact of a toxicant. The time is probably shorter for early life stages due to their smaller size as compared to later stage sizes (Petersen and Kristensen, 1998). The developing fish embryo and early larval stages have been shown to be especially sensitive indicators of many types of aquatic pollution (Devlin and Mottet, 1992; Pickering and Lazorchak, 1995; Hallare *et al.*, 2005). Sensitivity of embryonic stages to chemical-induced adverse effects is based on the occurrence of developmental events e.g. organogenesis (Honkanen, 2004). While there is an extensive literature describing the impact of heavy metals on Egyptian freshwater and marine adult fish (Awadallah *et al.*, 1985; Elnabawi *et al.*, 1987; Elsokkary and Muller, 1990; Abdel-Moneim *et al.*, 1994; Khallaf *et al.*, 1994; Elsokkary *et al.*, 1995; Ahmed, 1996; Mohamed *et al.*, 1998; Adham *et al.*, 1999; Hussein and Mekkawy, 2001; Rashed, 2001b; a; Adham, 2002), few studies have addressed the effect of the heavy metals on the embryonic and larval stages of fishes as bio-indicator (Lashein, 1996; Hamdy *et al.*, 2000; Mekkawy and Lashein, 2003).

1.3 The African catfish *Clarias gariepinus*

The African catfish *Clarias gariepinus* is among the most widespread freshwater fishes in Africa (Nguyen and Janssen, 2002). It inhabits tropical swamps, lakes and rivers (De Graaf and Janssen, 1996). During the last decade *C. gariepinus* was even introduced in Europe, Asia and Latin America for farming purposes (Verreth *et al.*, 1993). Female African catfish have fully developed ovaries containing ripe eggs whole year round, if kept in ponds once the water temperature remains above 22 °C. Fry and fingerlings of the African catfish are difficult to obtain in natural water. Alternatively, eggs and larvae can be obtained artificially by the induction of spawning via hormone injection (Huisman and Richter, 1987) using carp pituitary suspension (Hogendoorn and Vismans, 1980), human chorionic gonadotropin (Eding *et al.*, 1982), 17-hydroxy progesterone (Richter *et al.*, 1985) or synthetic luteinizing hormone-releasing hormone analogs (Huisman and Richter, 1987). In catfish culture in Asian and in African

countries, carp pituitary powder is most frequently used in artificial spawning. The economic importance of this species has increased tremendously in recent years as a result of its extensive use in aquaculture (De Graaf and Janssen, 1996). Furthermore, natural populations of *C. gariepinus* form a staple diet for many subsistence farmers throughout the African continent. In spite of the short developmental time of the African catfish and ease of culturing and year round reproduction there is a lack of information about its fertilization and the associated mechanisms and also about its embryonic and ontogenetic development. **Therefore**, studies were performed to clarify the fertilization mechanisms (chapter 3) and the embryonic development (chapter 4) to improve the knowledge about the early life history of *C. gariepinus* and to use the results of such studies for the discussion and interpretation of the toxicological investigations (embryo-toxic effects of lead nitrate).

Besides being an excellent candidate for aquaculture, *C. gariepinus* has also been used in fundamental research and for ecotoxicological studies (Nguyen *et al.*, 1997; Nguyen *et al.*, 1999; Nguyen and Janssen, 2002; Olaifa *et al.*, 2003). The use of only one developmental stage in toxicity testing is not enough for the risk assessment of chemicals. In spite of this, the use of different developmental stages of organisms in ecotoxicology research could be more informative (Honkanen, 2004). Also, selection of the appropriate biological markers for the monitoring metal-effect/low dose-response relationships is frequently a controversial issue. **Therefore, the present toxicological work** focuses on lead toxicity in different developmental stages of *C. gariepinus* (from fertilization to the end of the endogenous feeding period) considering different biological markers (biomarkers) comprising changes in the development and hatching rate, morphological and histological changes, biochemical changes (alteration of metabolic enzymes activity) and molecular changes (monitoring of DNA damage).

1.4 Outline of the work

The present work is presented in 7 chapters:

In chapter 1 a general introduction to the work was presented giving an idea about the problem of water pollution, heavy metals and lead in Egypt and it gives also some information about the test organism *C. gariepinus*. In addition to such general introduction, an extensive introduction to each specific issue treated in this dissertation will be given at the beginning of each chapter (chapters 3 to 7).

In chapter 2 a complete description of the materials and methods are presented.

In chapter 3 studies are described concerning the morphological variations of the egg envelopes before and after fertilization and the structure of spermatozoa using transmission and scanning electron microscopy. It also describes the process of chorion hardening and its biochemical basis.

In chapter 4 the early developmental stages from fertilization to the end of the endogenous feeding period are described focusing on the anatomical ontogeny of the selected organs.

In chapter 5 the effect of lead nitrate on the development and hatching success are investigated and lead-induced morphological and histological malformations in the embryonic stages are described.

In chapter 6 studies are described concerning the effect of lead nitrate on the activity of the metabolic enzymes G6PDH, LDH and PK during early development.

In chapter 7 investigations are presented describing the lead-caused DNA damage in embryos after exposure to lead nitrate by using the comet assay technique.

CHAPTER 2

Materials and Methods

2.1 Artificial reproduction of the African catfish *Clarias gariepinus*

Artificial reproduction of *C. gariepinus* took place in the hatchery of the Department of Fish Culture and Fisheries (Wageningen Agricultural University, The Netherlands). Spawners were selected according to the criteria described by De Graaf and Janssen (1996). Females (1-1.5 kg body weight) and males (1-3 kg body weight) were fed on a commercial pellet diet (3 % of body weight per day) and kept together in 800 l rectangular tanks containing tap water (conductivity 2000 $\mu\text{S}/\text{cm}$; pH \approx 7.5; Oxygen 90-95 % saturation; temperature 25 °C; photoperiod 12:12 Light: Dark). The tanks were the part of a recirculation system. Male spawners were anaesthetized and subsequently killed with 400 mg/l MS-222 (Tricaine methanesulfonate, Crescent Research Chemicals, Phoenix, Arizona, USA) buffered with 800 mg/l Sodium bicarbonate. Afterwards the testes were removed and sperms were pressed into a sterile dry Petri dish and immediately used for artificial reproduction. For the collection of eggs, ovulation was artificially induced by intra-muscular injection with 4 mg/kg body weight carp pituitary suspension (Cps, Stoller Fisheries, Spirit Lake, Iowa, USA.). 11-12 hours after the injection, fish were stripped and the eggs were collected in plastic containers; dry fertilization was performed. According to Balon (1975), the embryonic period comprises all stages from fertilization until the start of exogenous feeding including the egg stages (between fertilization and hatching) and the eleutheron-embryonal stages (yolk sac period) (Verreth *et al.*, 1992). In this work we apply this terminology.

2.2 Sampling for embryonic development:

The samples were collected at 30-min intervals for the first 24 hours and then every hour until hatching. Developmental time post fertilization was rounded to the nearest minute until the morula stage and then to the nearest hour. The time of fertilization here is the time of insemination.

2.3 Transmission Electron Microscope (TEM)

Pieces of the eggs and the testis were immediately fixed by immersion in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer for 24 h at 4 °C. The specimens were washed

in 0.1 M cacodylate buffer (pH 7.2) for 1-3 h and then post fixed in 1 % osmium tetroxide for 2 h. The tissue pieces were placed in propylene oxide for 60 min, then in pure Epon 812.

Tissues were semithin-sectioned at 1 μ m and stained with toluidine blue. Sections were examined by light microscope to identify different representative regions to be sectioned. Ultrathin sections were mounted in copper grids, stained with uranyl acetate and lead citrate (Bancroft and Stevens, 1982) and examined with TEM (JEOL 100, CXII) operated at 80 kv.

2.4 Scanning Electron Microscope (SEM)

Eggs before and after fertilization were fixed with 5 % glutaraldehyde in 100 mM phosphate buffer (pH 7.4, 4 °C) for 24 h. They were post fixed with 1.5 % osmium tetroxide for 2 h and washed four times with 100 mM phosphate buffer (pH 7.4). Some eggs were cut into halves with a fine razor. After slowly dehydrating with an ethanol series, the eggs were dried at 30-40 °C, glued to stubs coated with 20 nm of gold and viewed with SEM (GAOL, GSMS 400 LV) at 15 Kv.

2.5 Gel electrophoresis of chorion proteins

The isolated chorion of the fertilized and unfertilized eggs of *C. gariepinus* were dissolved in buffer containing 150 mM NaCl, 20 mM Tris, 10 mM EDTA, and 1 % SDS (Sodium Dodecyl Sulfate) with boiling at 100 °C for 5 min and centrifugation to remove undissolved remnants. One-dimensional SDS-PAGE (Poly Acrylamide Gel Electrophoresis) using 10 % acrylamide was performed according to the procedure of Laemmli (1970). The low molecular weight standards (Pierce, USA) were run concurrently and the protein molecular mass was determined using Gel-Pro Analyzer package (Media-Cybernetics, 1988).

2.6 Terminology of the chorion

There is considerable variation in the nomenclature used to describe the external membrane of the teleost egg. Commonly used terms for this outer covering of the egg include zona radiata, zona pellucida, chorion, radiate membrane, egg membrane, primary membrane, vitelline membrane, vitelline envelope, egg envelope, egg shell and egg capsule. In the present work, the term chorion is used according to Yamagami *et al.* (1992).

2.7 Experimental setup and sampling for toxicity tests

Fertilized eggs were divided into four groups, one control group (tap water) and three groups exposed to 100, 300 and 500 µg/l of lead nitrate. Exposure took place in 12 aquaria (20 cm * 20 cm * 5 cm) representing three replicates for each group. Lead nitrate standard solution (1000 mg/l, VWR (MERCK), Germany) was used for exposure. Temperature was kept at 24 °C.

Exposure started 6 h post-fertilization and the required embryos were collected at each sampling point, at 30 h post-fertilization (30h-PFS), 48 h post-fertilization (48h-PFS), 96 h post-fertilization (96h-PFS), 144 h post-fertilization (144h-PFS), and 168 h post-fertilization (168h-PFS). Thus, intervals represented 24, 42, 90, 138 and 162 hours of exposure time, respectively. 30 h-PFS was the only pre-hatching stage, since the hatching process started 40 h post-fertilization.

2.8 Morphological malformations

Four gross morphological malformations were observed; irregular head shape, pericardial edema (accumulation of a fluid in the pericardial cavity), yolk sac edema (accumulation of body fluid in the region of the yolk sac), notochordal defects [lordosis (dorsoventral curvature), kyphosis (ventrodorsal curvature), scoliosis (lateral curvature) and C-shape curvature] and two minor malformation were considered; finfold defects (reduction, blistering and necrosis of the fin) and reduction of body pigmentation. Malformed embryos were fixed for 24 h in 10 % formalin and the malformations were documented in using a dissecting microscope (Leica Wild MPS52) and a digital colore video camera (JVC).

2.9 Histology and histopathology

10 embryos from the control and lead exposed groups were fixed (at each sampling point) for 24 h in Bouin's solution. Fixed specimens were dehydrated and subsequently embedded in paraffin. Sagittal and transverse serial sections were cut at 5-7 µm, stained with haematoxylin and eosin (H&E) (gross histological) and Alcian blue (staining of chondrocytes ground substance of the embryonic skeleton). Stained sections were studied using a Leica DMRBE microscope and a digital 3 CCD colore video camera (Sony, AVT-Horn).

2.10 Determination of lead concentration

10 embryos were pooled at each sampling point, digested by means of a microwave (MLS-GmbH) after addition of 6 ml nitric acid and 2 ml hydrogen peroxide. Lead concentration was analysed according to German industrial standard, DIN 38406-6, (DEV, E6) with an Atomic Absorption Spectrometer 3300 Perkin Elmer using flame and graphite furnace technique. The results were calculated in microgram per gram wet weight ($\mu\text{g/g}$ wet wt).

2.11 Measurements of enzyme activities:

Due to the small size of the embryos, whole body homogenates were used for the measurement of the enzyme activities. Homogenisation of the whole animal is a reliable method providing a reasonable index of total enzyme activity in individuals whose body mass is predominantly composed of muscle tissue (Berges and Ballantyne, 1991; Lemos *et al.*, 2003). This procedure makes interpretation of the results more equivocal than investigations in older life stages when specific organ preparations can be used (Segner and Verreth, 1995).

The activities of the selected enzymes were measured according to a modified protocol based on Hardewig *et al.* (2004). Four embryos from the control and lead exposed groups (at each sampling point) were pooled and pulverized under liquid nitrogen and about 100 mg of ground tissue powder was added to 5 vol. of buffer (50 mM Tris, pH 7.4, 1 mM EDTA and 2 mM MgCl_2). Tissue was homogenized briefly with an Ultra-Turrax (temperature was maintained at 4 °C during homogenisation). The homogenate was centrifuged for 15 min at 10,000 Xg and 4° C and supernatants were used for the enzyme activity assay. Activities were determined in the supernatant with a plate reader (spectra Flour Plus, Tecan) at a wavelength of 340 nm and 25 °C. G6PDH was determined in extraction buffer containing 100 mM Tris/HCl (pH 7.6), 0.5 mM NADP, 3 mM Glucose-6-Phosphate, 6 mM MgCl_2 and 5 mM Maleinimide. The reaction mixture contained 50 mM imidazole, 0.2 mM Pyruvate-Na and 0.15 mM NADH for LDH. For Pyruvate Kinase (PK) 50 mM Imidazole, 5 mM ADP, 100 mM KCL, 10 mM MgCl_2 , 0.15 mM NADH, 0.1 mM fructose 1-6 Phosphate, 5 mM Phosphoenolpyruvate and 25 U/ml LDH was used as extraction buffer. The catalytic activity content for the selected enzymes was calculated in terms of units/g protein (Bergmeyer *et al.*, 1983).

2.12 Comet assay

The comet assay was performed according to a modified protocol based on Jarvis and Knowles (2003). Embryos were macerated with forceps in 25 μ l Ca^{2+} , Mg^{2+} -free phosphate buffer saline. 50 μ l 1 % low melting point (37 °C) agarose was added. The total volume (75 μ l) was layered onto a frosted microscope slide, previously coated with 1 % normal melting point agarose. The slide was incubated at 4 °C for 15 min to allow solidification and was subsequently coated with an additional layer of 1 % low melting point agarose. After solidification at 4 °C for 20 min, the embedded cells were lysed in lysing buffer [2.5 M NaCl, 100 mM NaEDTA, 10 mM TRIS base, pH 10, 1 % TritonX-100, 10 % DMSO] at 4 °C for 120 min. After 30 min incubation in electrophoresis buffer [300 mM NaOH, 1 mM EDTA, pH \geq 13] electrophoresis was carried out at 20 V and 300 mA for 30 min. Subsequently, neutralisation was performed in three washing steps in 0.4 M Tris-HCl (pH 7.5). The slides were fixed in 100 % ethanol (5 min), rehydrated in ultra-pure water (5 min) and stained with ethidium bromide solution [20 μ g/ml] for 10 min, followed by a final washing step in ultra-pure water. Analysis was performed with a Zeiss Axioplan2 fluorescence microscope (x200) and a digital 3 CCD color video camera (Sony, AVT-Horn) using the TriTek Comet image analysis software. Considering 32 nuclei, three replicates per treatment were analysed at each sampling point. Percentage of DNA in the tail assessed as optical intensity at 515-560 nm (% TDNA), percentage of DNA in the head (% H DNA), tail length (TL) and tail moment (TM = TL * % TDNA) (Fig. 32) were calculated for each nucleus. Calculations were averaged per replicate.

For qualitative evaluation, nuclei were categorized according to the degree of damage (using % TDNA) based on the criteria of Anderson *et al.* (1994) and Mitchelmore and Chipman (1998b) into five classes: undamaged (% TDNA \leq 10 %), low damaged (10 % < % TDNA \leq 25 %), median damaged (25 % < % TDNA \leq 50 %), highly damaged (50 % < % TDNA \leq 75 %) and extremely damaged (75 % < % TDNA) (Fig. 34).

2.13 Statistical analysis

For frequency assessment of the morphological malformations, only the number of deformed embryos was considered in each category. Data were expressed as mean \pm SD (n=3). Multiple comparison was performed with non-parametric Bonferroni test. Significance was accepted at $p < 0.05$ (StatSoft, 1995).

For enzyme activities the basic statistics, means, standard error and range were estimated. The patterns of variation due to developmental stages and lead-doses and their interaction were studied by two-way analysis of variance using SPSS package (SPSS, 1998) at 0.05 significance level. Levene's test of equality of error variance of the dependent variables was applied with rejection of the null hypothesis for raw, log-transformed and SQRT-transformed data. So, the homogeneity of variance was assumed for raw data. The model considered is: intercept +age +treatment +age * treatment (adjusted R² was 0.966, 0.965 and 0.943 for G6PDH, LDH and PK respectively). A further design (age + treatment +age * treatment) exhibited similar significant pattern of variations for the main effect of age and treatment factors and their interaction ($P < 0.0001$) (Adjusted R² was 0.993, 989 and 0.973 for G6PDH, LDH and PK respectively). The pattern of variations also was recorded by one-way analysis of variance revealing significant difference within the development stages and treatments ($P < 0.0001$); no homogeneity of variance even with log- and SQRT-transformations was recorded. Tukey-HSD test was considered for multiple comparisons. Moreover, the Dunnett-*T* test was applied measuring the control against other treatments in each developmental stage.

For the comet assay the mean \pm standard error were considered. The patterns of variation due to developmental stages and lead-doses and their interaction were studied by a one way analysis of variance ANOVA considering non-parametric Bonferroni. Significance was accepted at $p < 0.05$ (StatSoft, 1995). The dose that damaged 50 % of the nuclei (IIC50) was established by Probit analysis (SPSS, 1998).

CHAPTER 3

Fertilization and associated morphological and chemical variations of the egg surface and envelopes

3.1 Introduction

More than 50 % of all vertebrate species comprise teleosts (Baldacci *et al.*, 2001). An important feature of the teleosts evolutionary success is their reproductive system, which must be functional under various aquatic environmental conditions. However, much of the existing knowledge of the mechanisms involved in teleost fertilization is based on few small model species such as zebrafish, medaka and bitterling which have no real commercial value (Coward *et al.*, 2002). Research is required to address mechanisms involved in the fertilization in species of commercial value, which represent a cornerstone of the field of aquaculture and the associated biotechnology.

Interspecific differences in the microstructure of the chorion (egg envelope) of teleost fishes have been recognized for almost 30 years (Merrett and Barnes, 1996). Such differences not only have been used to identify eggs in plankton samples (Merrett and Barnes, 1996) but are also potentially useful taxonomic characters (Gill and Mooi, 1993; Johnson and Brothers, 1993; Britz *et al.*, 1995; Chen *et al.*, 1999; Britz and Breining, 2000). Chen *et al.* (1999) concluded that the ultrastructural features of the egg envelope are helpful in species identification of distantly related species, but not of closely related ones. Merrett and Barnes (1996) confirmed Marshall (1973) suggestion that egg envelope ornamentation is a family characteristic.

The chorion structure and its chemical constituents are the end product of different evolutionary trends, adaptation processes and environmental factors (Yamagami *et al.*, 1992; Celius and Walther, 1998; Quagio-Grassiotto and Guimaraes, 2003). Hyllner *et al.* (2001) concluded that the chorion proteins from the vertebrate groups of fishes and their amphibian, avian and mammalian counterparts share a common ancestry and form a unique group of structural proteins.

The fertilization process of a fish must be studied and described on the light of reorganization of the chorion at fertilization, its physiological roles, the dynamic

changes of the egg cortex, the secretory functions of cortical granules, mechanisms of sperm-egg interactions, and mechanical blocking to polyspermy (Lonning and Hagstrom, 1975; Hart, 1990; Yamagami *et al.*, 1992; Griffin *et al.*, 1996; Merrett and Barnes, 1996; Linhart and Kudo, 1997; Chen *et al.*, 1999; Britz and Breining, 2000; Coward *et al.*, 2002). Literature dealing with *C. gariepinus* chorion and fertilization and the associated mechanisms is very rare. Only two articles (Riehl and Appelbaum, 1991; Wenbiao *et al.*, 1991) are at hand. They studied the structure and the position of micropyle with respect to fertilization. Also they described the attachment mechanism of the fertilized eggs. They did not study the effect of fertilization on morphological changes of the egg envelope or on the molecular structure of the chorion. So the current study was undertaken to cover some of these points. The present work aims to elucidate the morphological variations of the chorion of *C. gariepinus* egg before and after fertilization. It also aims to emphasize the process of chorion hardening and the biochemical composition of the chorion, in addition to the structure and behavior of spermatozoa on the chorion surface using transmission and scanning electron microscopy. The micropyle shape and its closure, the micropyle-like depressions, the folded chorion and the polyspermy prevention and fertilization mechanisms were also considered and discussed in the light of the available information.

3.2 Results

3.2.1 The ripe testes and the sperm structure

The testes of *C. gariepinus* are composed of divided lobules separated from one another by interlobular connective tissue. The testes in a ripe stage display active spermatogenesis with various stages (Fig. 1a, b). The seminiferous lobules are highly distended by spermatozoa (Fig. 1c, d). The spermatozoa of *C. gariepinus* were tightly packed in the lumen of the testes lobules (Fig. 1c, d). Spermatozoon consisted of a head, midpiece and very long tail (Fig. 2a). The head region contained the nucleus which consisted of variable electron dense granular chromatin materials. The head region was relatively triangular or rectangular. The midpiece had an inverted conical shape forming an oval shaped structure with the head (Fig. 2b). The head-midpiece surface showed irregularity (surface with irregular folding, Fig. 2b). Figure 2 (c, d) showed other related structures especially the flagellum structure in cross and longitudinal sections. The anatomy of spermatozoa seemed to be adjusted with the diameter of the inner aperture of the micropylar canal.

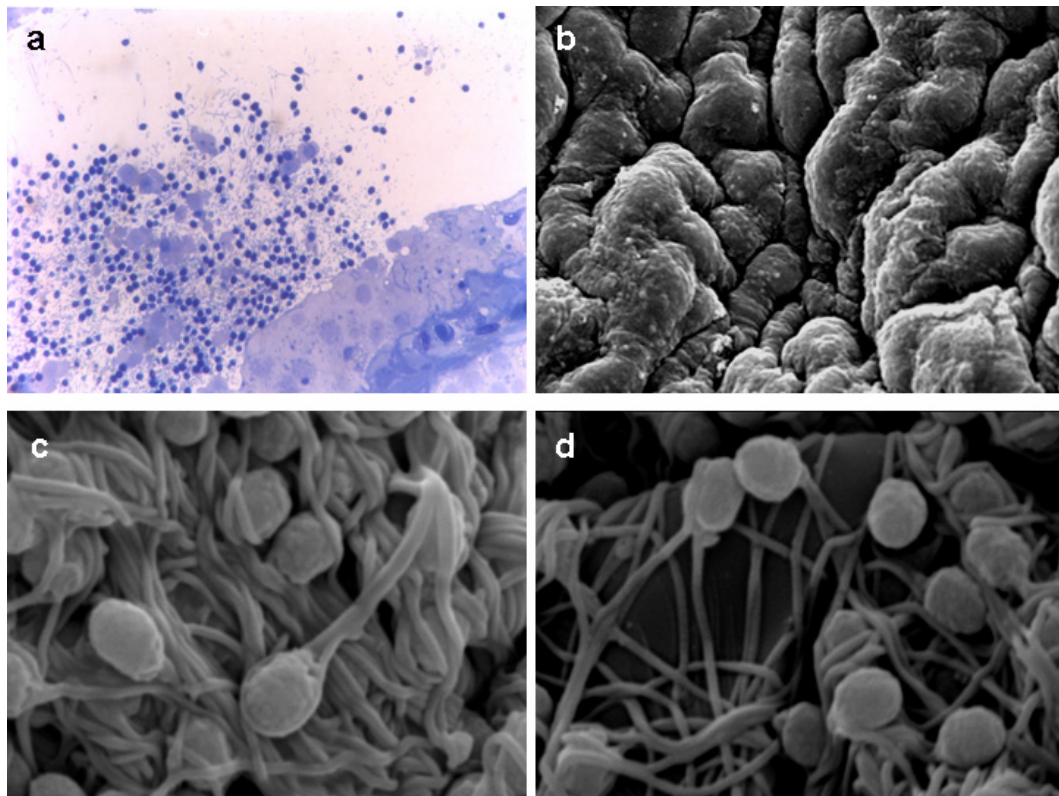


Fig. 1. The rip testis of *Clarias gariepinus* in semithin sections (a: x1000) showing spermatozoa and other developmental stages. SEM micrographs of the ripe testis (b: x3500), showing interstitium and testis lobulated internal structure. Spermatozoa in the lobules of the rip testis (c, d: x10000).

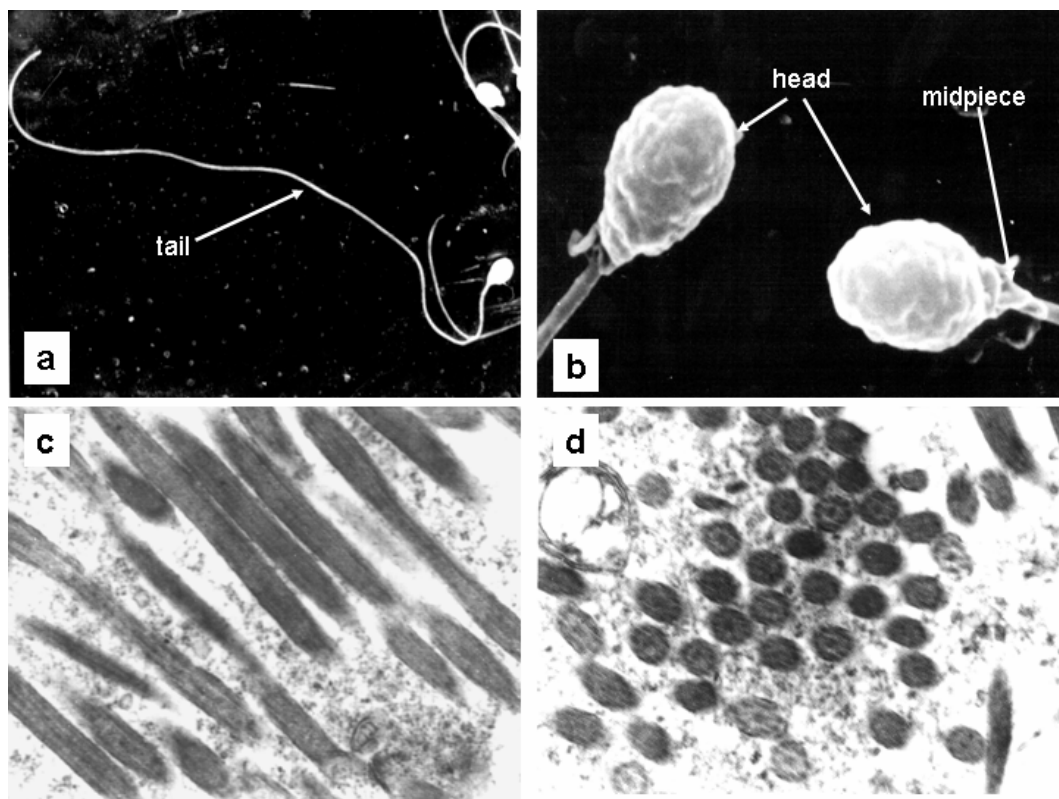


Fig. 2. Spermatozoa of *Clarias gariepinus* in semen as revealed by SEM (a: x3500, b: x20000). (a) Showing a sperm with very long tail, (b) showing the irregularity of the surface of the sperm head and midpiece, (c, d: x20000) represent longitudinal and transverse sections of the flagellum of the sperm, respectively

3.2.2 The ripe oocyte

The ripe oocyte had four distinct layers: an outermost follicular layer (outer theca + inner granulosa layer), a median zona radiata (the chorion) and an inner oolemma or oocyte plasma membrane (Fig. 3a, b). The chorion of the ripe oocyte was a single layer (sections shown in Fig. 3c, d, e).

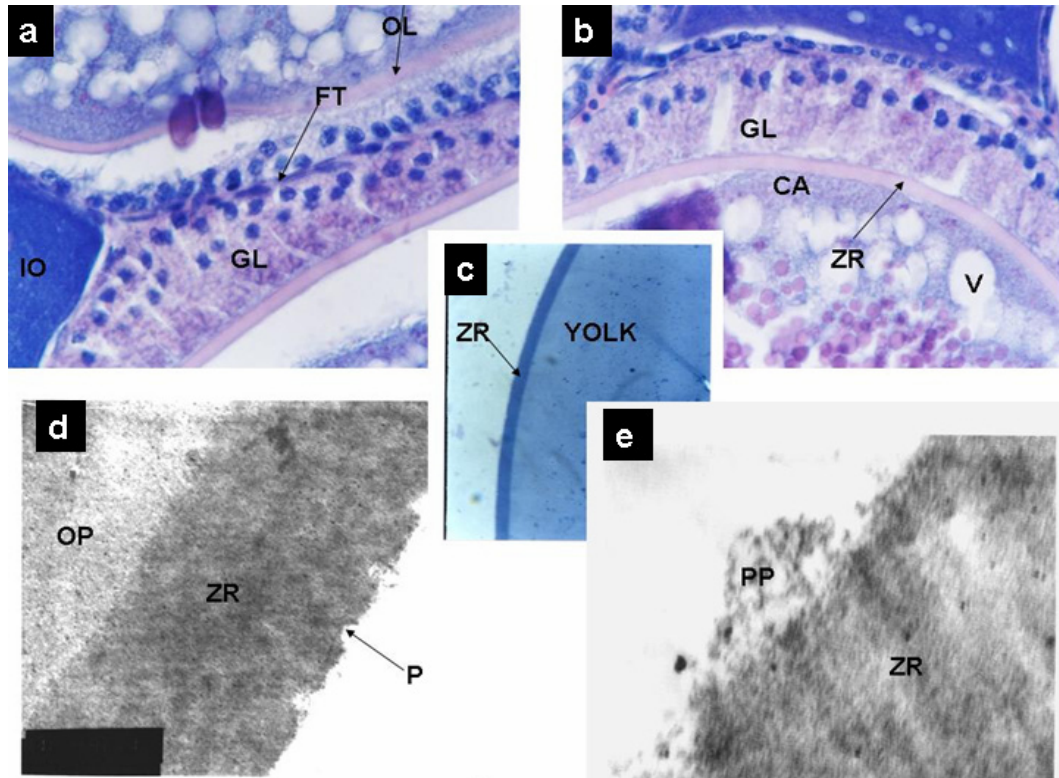


Fig.3. The ripe ovary of *Clarias gariepinus* in different cross sections (a, b: x1000) showing follicular theca (FT), granulosa layer (GL), single-layered zona radiata (ZR) and oolemma (OL). CA: cortical alveoli, IO: immature oocyte, V: vacuole. The zona radiata of unfertilized egg as seen in semithin (c: x1000) and ultrathin (d, e: x10000, 40000, respectively) sections. Note the faint radiated ZR in e. P: pore, PP: pore plug, OP: ooplasm.

3.2.3 Egg surface

The outer surface of the unfertilized egg of *C. gariepinus* is smooth (Fig. 4a) whereas the fertilized egg acquired a network of projection 1-h post fertilization on the vegetal hemisphere (Fig. 4c-f). On the vegetal pole, such projections formed a base like circle (Fig. 4f). At high magnification (X7500), some of these elongated projections had a dentate lateral process (Fig. 4d). The network projections seemed to be inserted in the chorion. White spots were recorded within the network projections (Fig. 4c, e). Different patterns of ornamentation on the egg surface were evident (Fig. 5).

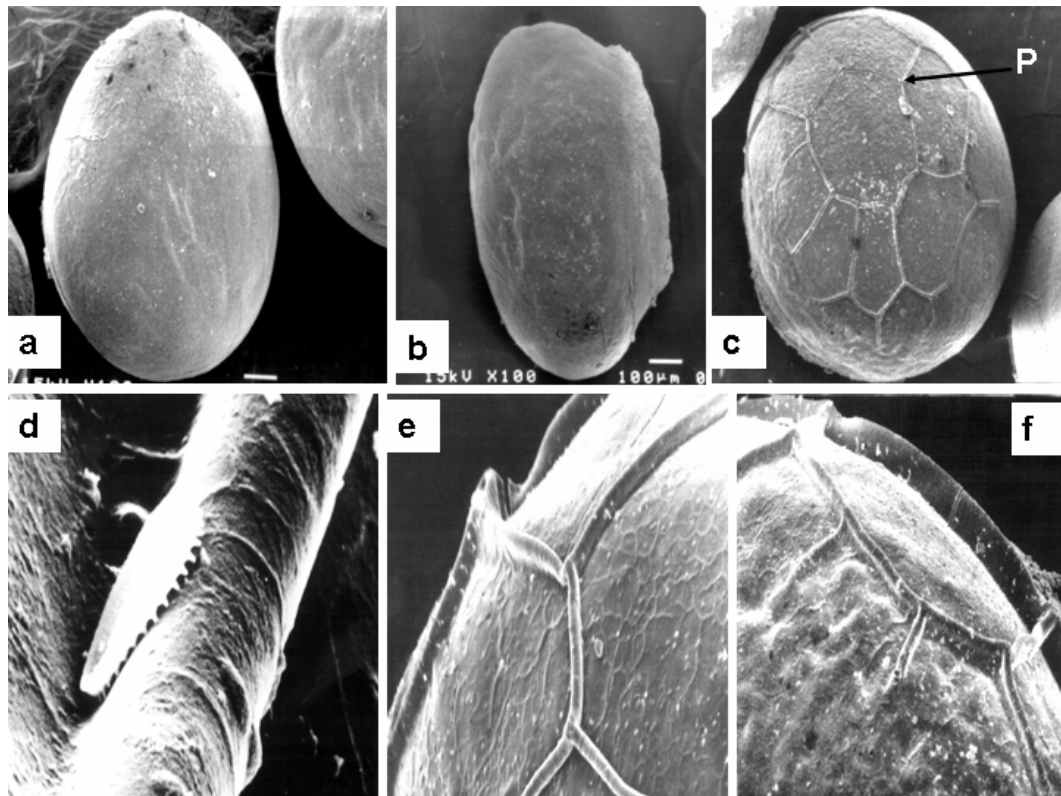


Fig. 4. The ovulated (a) and fertilized eggs of *Clarias gariepinus* (b, (c+d), f at $\frac{1}{2}$ -h, 1-h, 4-h postfertilization stages, respectively). Network projections (P) on the vegetal hemisphere were formed about 1-h postfertilization (a, b, c: x100; d: x7500; e: x500; f: x350).

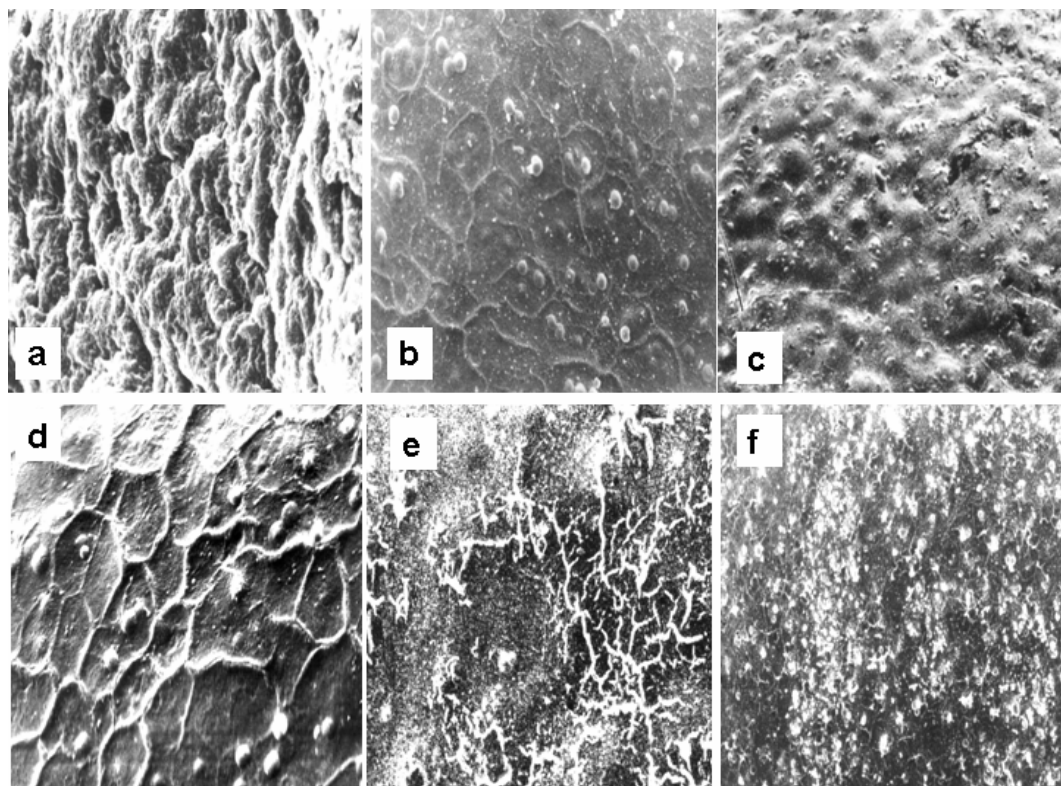


Fig. 5. Ornamentation of different regions of the egg surface of *Clarias gariepinus* at $\frac{1}{2}$ -h (a, b, c) and $1\frac{1}{2}$ -h (d, e, f) postfertilization stages (a: x3500, b: x1500; c: x750; d: x2000; e: x2000, f: x750). a: lobulated ornamentation, b: debris-like dots, c: pore bluges, d: debris-like batches, e: tubercles, f: reticulated ornamentation.

Such ornamentation has been represented as irregularly lobulated ornamentations (Fig. 5a), debris-like dots (Fig. 5b), irregularly distributed pore bulges (Fig. 5c), debris-like batches (Fig. 5d), tubercle and/or reticular (Fig. 5e), and partially reticulated debris (Fig. 5f). These patterns were recorded in different regions and at different postfertilization times. For example, irregular lobulated ornamentation, debris-like dots and irregularly distributed pore bulges were recorded at ½-h postfertilization, whereas, the batches, the tubular and the reticular ornamentations were observed at 1-h postfertilization.

The ornamentation varied from the germinal disc region to the vegetal hemisphere (Fig. 6). Moreover, the pattern of ornamentation varied with the progress in embryonic development since hairs and depressions appeared on the animal hemisphere in comparison with the vegetal one (Fig. 6d). Further changes were recorded at 30 h stage (Fig. 6e).

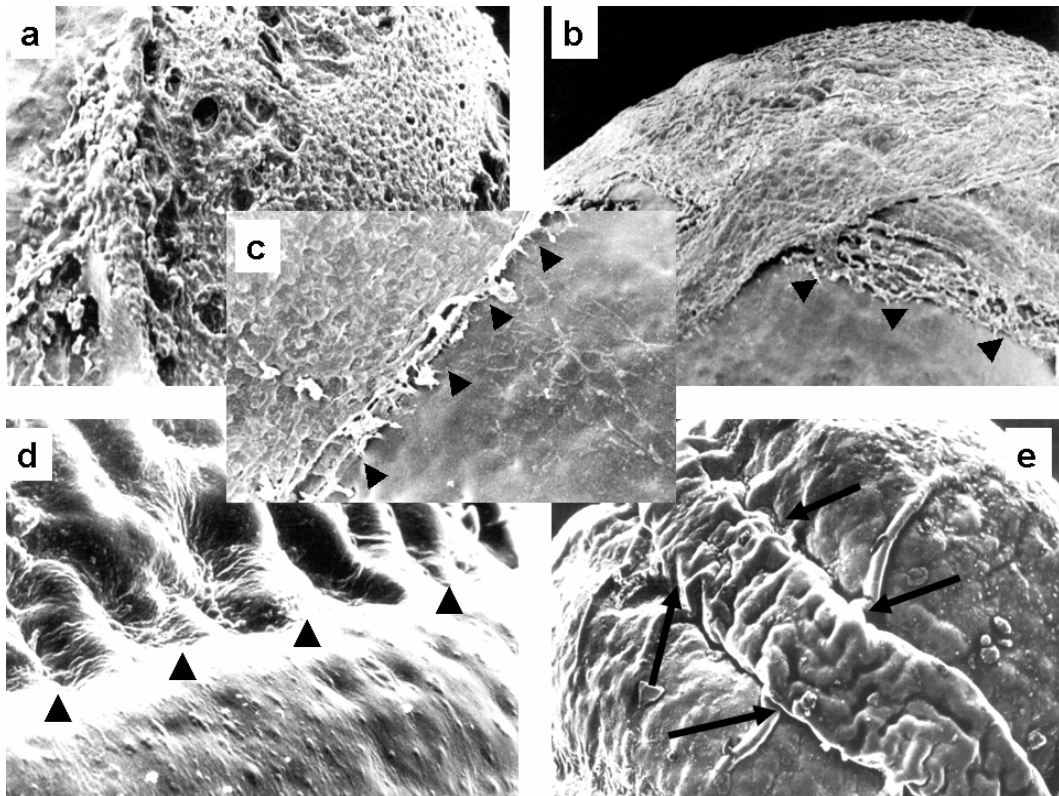


Fig. 6. Ornamentation on the micropylar disc (a, at ½-h postfertilization), the separation line (head arrow) between animal and vegetal hemispheres (b,c, at 1-h postfertilization; d, at 9-h postfertilization) and the region of *Clarias gariepinus* embryo attachment (arrow) (e, at 30-h postfertilization) (a: x350; b,e: x200; c,d: x750).

3.2.4 The micropyle

The micropyle consisted of a funnel-shaped vestibule from the bottom of which a cylindrical micropylar canal extended (Fig. 7a, b). The shape of the chorion around it appeared to facilitate the movement of spermatozoa toward the micropyle. With

development progress the micropyle continued to narrowing with the formation of the micropylar disc (Fig.7c, e). The network of projections on the vegetal hemisphere leads to the conclusion that these projections might represent another attachment mechanism. In our fertilization experiments of *C. gariepinus*, the animal pole was usually directed upward.

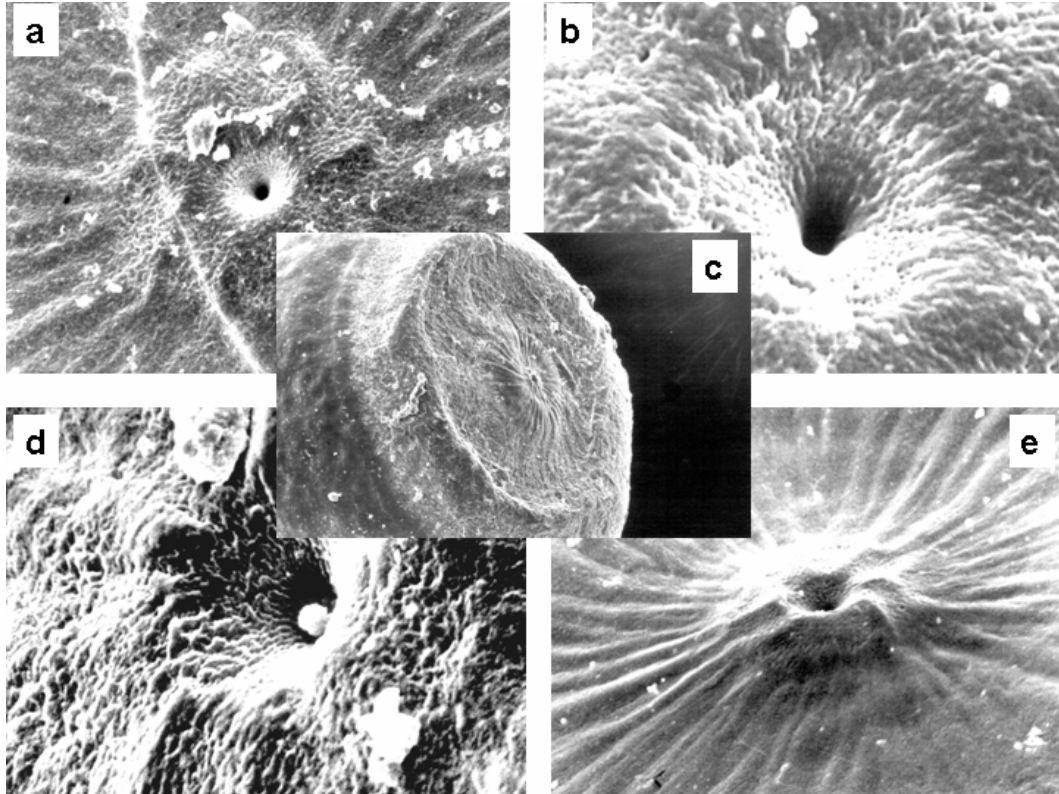


Fig. 7. The micropylar apparatus of *Clarias gariepinus* at different magnifications (a-b). Note ornamentation specific to the micropylar regions. The spermatozoon inside the micropylar canal (d, at ½-h postfertilization) and the formation of micropylar cone (c, e, at ½-h and 1½-h postfertilization, respectively) (a: x1500; b: x5000; c: x150; d: x3500; e: x1000).

The spermatozoa move along the surface of the chorion and enter the micropyle or any micropyle-like depression in a directed fashion (Fig. 8). Receptor of various sources or motility stimulating factors of *C. gariepinus* must be involved in aggregating spermatozoa. Accordingly, the multiplicity of micropyle-like depression was more beneficial for trapping sperm without the formation of cytoplasmic processes. This means the spermatozoa are oriented inherently toward any depression on the chorion surface in addition to the trapping mechanism on the chorion fibers (Fig. 9a, b).

3.2.5 The chorion structure and fertilization

The chorion of ovulated eggs consisted of one layer under the light microscope and TEM (Fig. 3c, d). The single-layered chorion of the unfertilized egg had faint striation (Fig. 3e). Directly after fertilization, the single zona radiate was differentiated

into zona radiata externa (ZRE) and zona radiata interna (ZRI) (Fig. 9c). At $\frac{1}{2}$ h and 1 h postfertilization stages, the chorion was differentiated into three

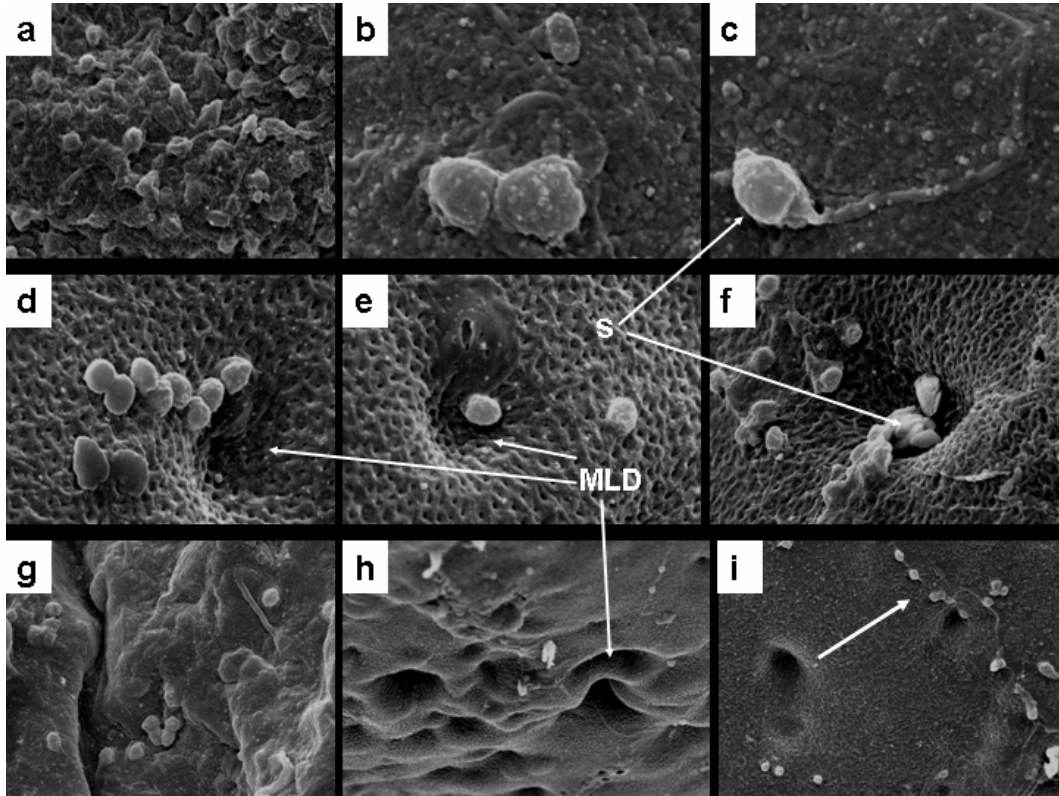


Fig. 8. Spermatozoa trapped on the surface of *Clarias gariepinus* eggs in higher number (a) with different white secretion on their heads and tails (b, c). Spermatozoa on the outer surface of the micropyle-like depressions at the vegetal pole (d-f). Note the ornamentation of the chorion in this region. The micropyle-like depressions and the folded chorion at the vegetal pole (g-i) with spermatozoa directed in lines (arrow). (a-i) 30 seconds postfertilization (a, i: x2000; b, c: x15000; d,e: x7500; f: x5000; g: x3500; h: x1500). S: spermatozoa, MLD: micropyl like dipression.

layers (Figs. 10, 11), the double-layered coat (DLC, Fig. 11c, e), the middle layer, the zona radiata externa (ZRE) and the inner most layer, the zona radiata interna (ZRI) which was half the thickness of ZRE. The pore canals were expressed in both ZRE and ZRI. These pore canals and striations were more prominent in ZRI (Fig. 10 a-d). Following ZRI, there was an electron opaque layer (Fig. 11a-d). This latter unnamed layer invaded the perivitelline space (PVS) and came into contact with the oolemma.

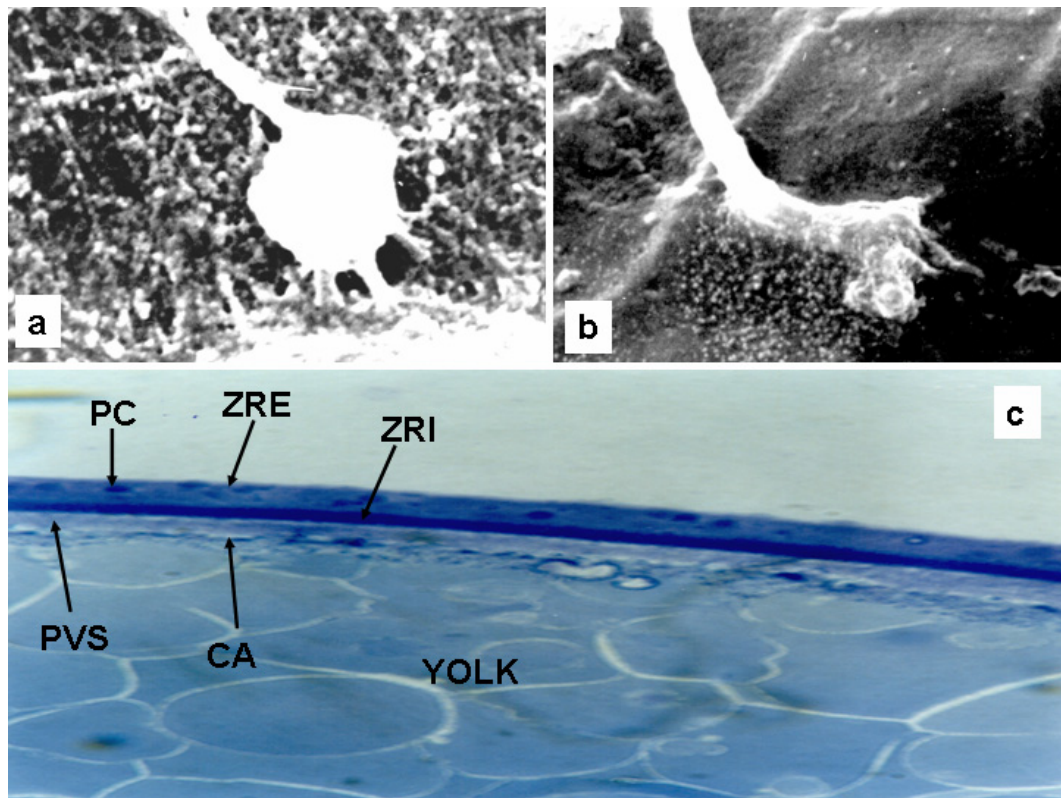


Fig. 9. Spermatozoon embedded by head on the egg surface (a: x20000) of *Clarias gariepinus* (b: x100000) with the chorion surface microvilli (a at 10 sec, b at 1 h postfertilization). Semithin sections of the fertilized eggs showing differentiation zona radiata (ZR) into zona radiata externa (ZRE) and interna (ZRI) after fertilization (c: x1000). CA: cortical alveoli, PC: pore canal, PVS: perivitelline space.

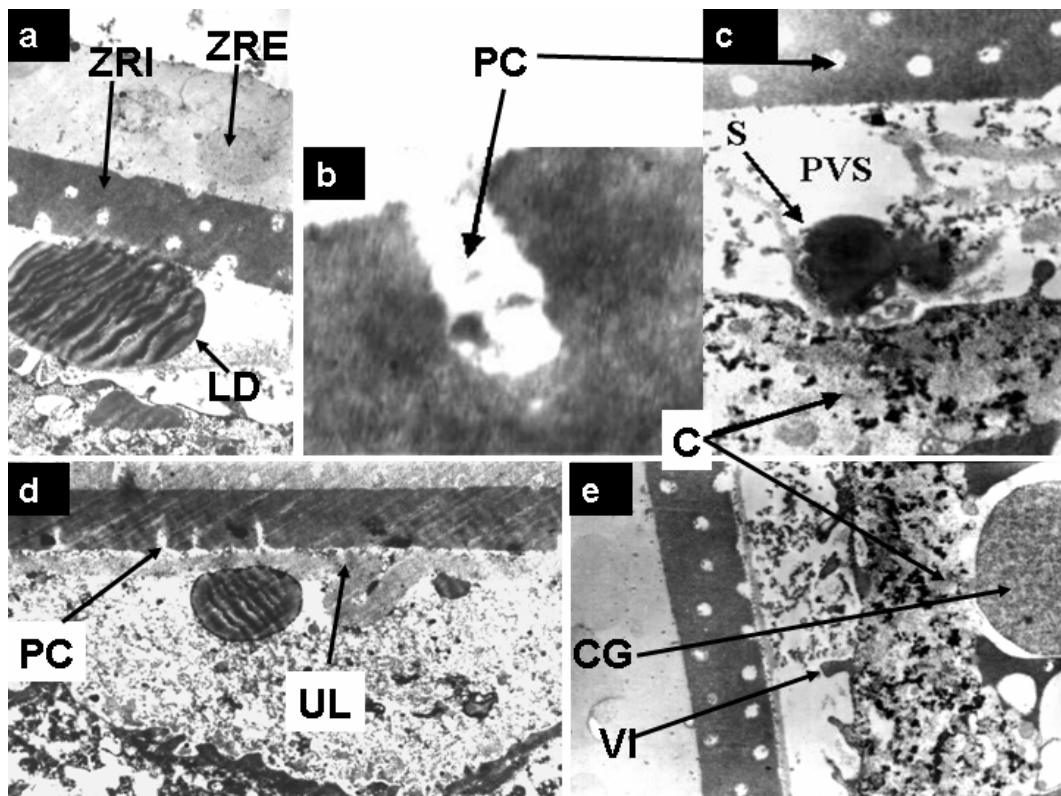


Fig. 10. Ultrathin sections of the chorion and the outer layer of the ooplasm of the fertilized eggs of *Clarias gariepinus* (a: x6700; b: x40000; c: x8000; d: x4000; e: x5000) (1-h postfertilization). PC: pore canal, UL: unnamed layer, CG: cortical granule, C: cortex, VI: villi, S: secretion, LD: lipid, PVS: perivitelline space.

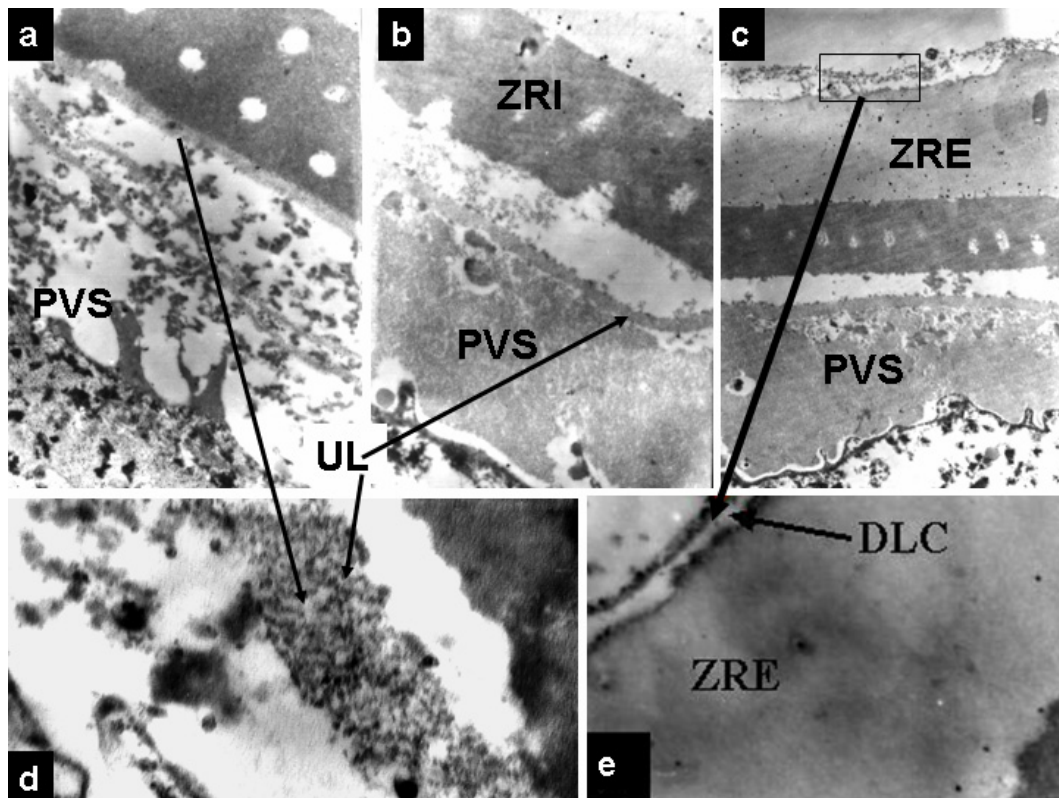


Fig. 11. Ultrathin sections of the outer layers of the fertilized eggs of *Clarias gariepinus* showing the double-layered coat (mucous coat, DLC) and the unnamed layer (UL) and UL-course in relation to ZRI and the plasma membrane (a,b: x10000; c: x5000; d: x40000; e: x20000) (1-h postfertilization). PVS: perivitelline space. ZRE: zona radiata externa. ZRI: zona radiata interna.

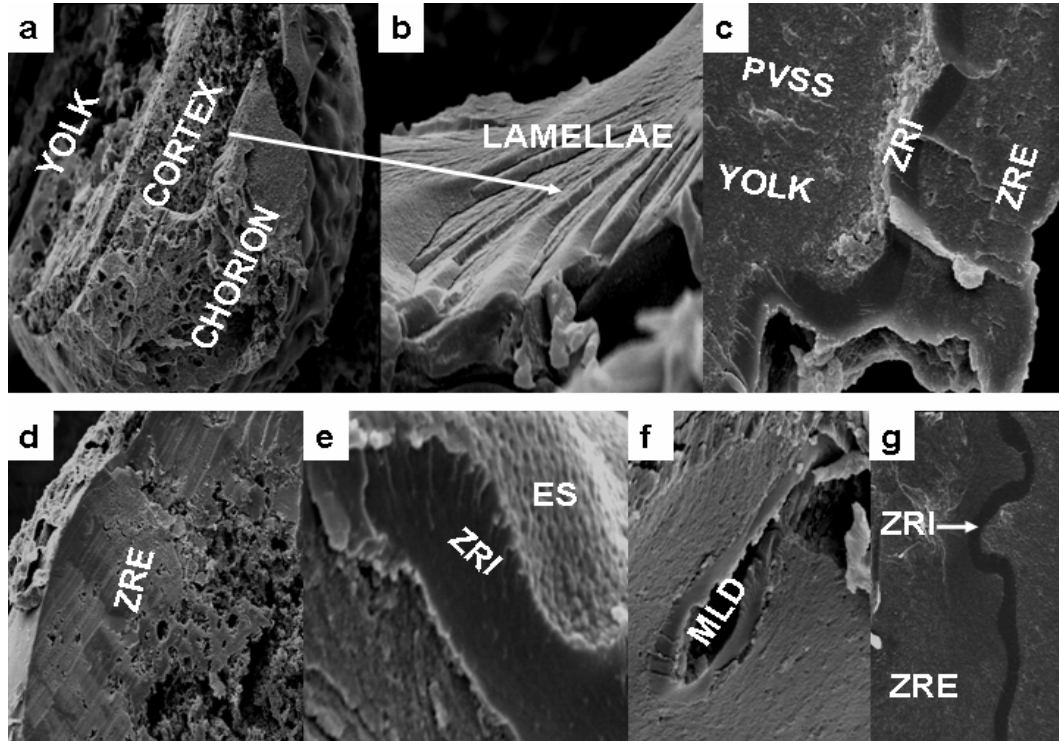


Fig. 12. Fractured parts of the fertilized eggs of *Clarias gariepinus* showing the chorion layers and the cortex. (a, b) Lamellar nature of zona radiata interna (ZRI) (a: x150; b: x5000). (c) Showing the tubular structure of ZRI and to some extent zona radiata externa (ZRE) and secretions in PVS (PVSS) (x2000), (d) Showing the great thickness of ZRE in the animal region (x350). (e, f) Disappearance of ZRE in the vegetal pole and at micropyle-like depression (MLD) after fertilization (e: x10000; f: x2000), note the tubular nature and the brushed inner side ZRI in e. (g) The thickness-relationship between ZRE and ZRI in the micropylar region (x1500). ES: egg surface.

The three layers of the chorion identified by TEM were detected by SEM with elucidation of their pore canals (Fig. 12a-c). The ZRE showed variability in its thickness in comparison with the ZRI on the same egg, especially in the regions of the the animal pole (Fig. 12d) and micropylar disc (Fig. 12g). The ZRE disappeared completely in the micropyle, in the micropylar-like depression and in the vegetable pole (Fig. 12e, f). The lamellar and tubular nature of ZRI was evident (Fig. 12a, b).

The current postfertilization morphological changes of the chorion were associated with cortical reactions and cortical alveoli discharge in the perivitelline space (Fig. 13). TEM has shown that the cortical cytoplasm of the eggs contained membrane-limited cortical granules with an internal matrix of varying electron density (Fig. 13).

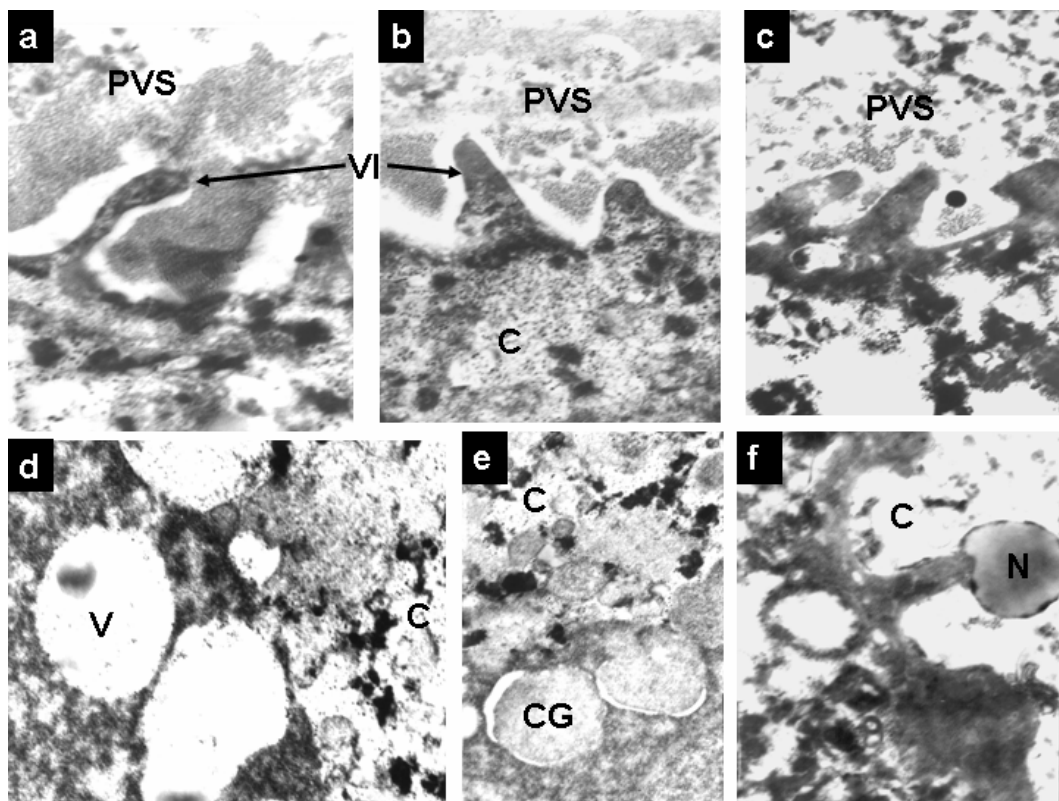


Fig. 13. Ultrathin sections in the cortex (C) of the fertilized eggs of *Clarias gariepinus* showing cortical alveoli, granules (CG) and their secretion in the perivitelline space (PVS) (a: x27000; b,c,f: x20000; d,e: x14000) (1-h postfertilization). N: nucleus, V: vacuole, VI: villi.

Four protein subunits of the chorion of the ovulated egg were identified by SDS-PAGE: three proteins of low molecular weights (MW) and one protein of relatively higher MW that had the highest percentages, (29.8-47.89 %) (Table 1). Hardening of ovulated egg resulted in no or minor variations in the MW. Fertilization influenced the chorion proteins of the first three categories (108.93 to 129.7-133.6; 31.5-33.7 to 35.6-

36.4; 25.3-26.9 to 28.3-29.2 KDa, respectively). The results of SDS-PAGE of chorion proteins subunits can be compared with those of other teleost fishes (Table 2).

Table 1. The chorion protein subunits (bands) identified by SDS-PAGE in the ovulated (OE), hardened OE, 1-minute post fertilization (MPF), and 1-hour and 2-hour post-fertilization (HPF) eggs of *Clarias gariepinus*. MW: molecular weight.

	MW	1		2		3		4		5	
Lanes	MW-Marker	Hardened OE		OE		1-MPF		1-HPF		2-HPF	
Bands	MW	MW	%	MW	%	MW	%	MW	%	MW	%
r1	223.0										
r2						133.6	43.1	132.6	47.0	129.7	29.8
r3	110.0	108.9	38.6	108.9	47.8						
r4	81.6										
r5	46.8										
r6						36.4	31.5	35.6	24.6	35.9	35.1
r7	31.8	31.5	32.0	33.7	26.8						
r8						28.3	25.4	28.8	12.0	29.2	15.0
r9	24.8	25.3	16.0	26.9	12.6						
r10	16.5	16.0	13.4	17.0	12.8			17.0	16.4	17.0	20.1

Table 2. The molecular size of protein subunits of different fish based on SDS-PAGE.

Fish species	Chorion protein subunits	Author (s)
<i>Oryzias latipes</i>	150, 77-73 and 49 KDa 132, 114, 62 and 61 KDa	Iwamatsu <i>et al.</i> (1995)
<i>Oryzias latipes</i>	76, 74 and 49 KDa	Hamazaki <i>et al.</i> (1987a, b; 1989)
<i>Gadus morhua</i>	78, 54, 47 KDa 109 and 98 KDa	Oppenberntsen <i>et al.</i> (1992)
<i>Oncorhynchus mykiss</i>	60, 55 and 50 KDa	(Hyllner <i>et al.</i> (1991; 2001)
<i>Dicentrarchus labrax</i>	44-250 KDa (several peptides)	Scapigliati <i>et al.</i> (1994)
<i>Epinephelus malabaricus</i>	97 and 76 KDa	Chiou <i>et al.</i> (2004)
<i>Carassius auratus</i>	At least 20 proteins (30-250 KDa) (classified in 5 principle classes-those of 40-60 KDa region represent the major class). Glycoprotein components (46, 60, 84, 110 KDa)	Cotelli <i>et al.</i> (1988)
<i>Chionodracco hamatus</i>	200, 92, 80, 68, 46 and 40 KDa	Baldacci <i>et al.</i> (2001)
<i>Coregonus lavaretus</i>	17-80 KDa (7 peptides)	Scapigliati <i>et al.</i> (1995)
<i>Danio rerio</i>	116, 97, 50 and 43 KDa	Bonsignorio <i>et al.</i> (1996)
<i>Clupea pallasii</i>	117& 48-54 KDa (90-95 % of the polypeptides)	Griffin <i>et al.</i> (1996)
<i>Gasterosteus aculeatus</i>	19.4, 36.7, 39.4, 42.9, 46.1, 53 KDa	Deung <i>et al.</i> (1999)
<i>Salmo salar</i>	60, 55 and 50 KDa	Oppenberntsen <i>et al.</i> (1994)
<i>Salmo salar</i>	66, 61 and 55 KDa	Celius and Walther (1998)

3.3 Discussion

3.3.1 The ripe testes and the sperm structure

As in most teleostean fishes, the testes of *C. gariepinus* are composed internally of intricately divided lobules separated from one another by interlobular connective tissue. The convoluted closely packed seminiferous lobules are similar to the seminiferous tubules of mammalian testes (Rizkalla, 1960). The latter author described the interstitium between the seminiferous lobules consisting of connective tissue fibers and cells, blood and lymph vessels, nerves, pigment cells and interstitial cells. The testes of *C. gariepinus* in a ripe stage displayed various stages of active spermatogenesis. The spermatozoon consisted of a head, midpiece and very long tail. The midpiece forming with the head an oval shaped structure with irregular surface. The irregular inverted conical shape of the midpiece reflected the increased number of mitochondria. The mitochondria were distributed separately around the flagellum. These findings were in contrast to those reported by Mansour *et al.* (2002), who stated that several single mitochondria were fused with each other and formed a complex chondriosome. It was difficult to postulate such chondriosome structure with the reticulate structure of the midpiece of *C. gariepinus*. The anatomy of spermatozoa is adjusted with the diameter of the micropylar canal. This condition was indicated by Linhart and Kudo (1997) as contributing to the prevention of polyspermy.

3.3.2 The ripe oocyte

Ripe gonads were characterized chiefly by migration of the nucleus toward the animal pole (Zaki *et al.*, 1986). The ripe oocyte of *C. gariepinus* had four distinct layers: outer theca, inner granulosa layer, the chorion and inner oolemma. Zaki *et al.* (1986) confused the terminology of the layers surrounding the oocyte since they referred to the outer layer (zona granulosa) as chorion and the inner layer as zona radiata (with clearly discernible pores). Rizkalla (1960) referred to 5 layers encircling the ovarian ripe oocyte of *C. gariepinus*: theca folliculi, membrana propria folliculi, follicular epithelium, definitive membrane proper (zona radiata with faint striation) and the zonoid layer (peripheral cytoplasm). The second layer of Rizkalla (1960) was not recorded in the present work. The chorion of the ovarian ripe oocyte consisted of a single layer. Consistent with the present result, Al-Absawy (2004) reported a single-layered chorion of the ovarian oocyte of *Trachinotus ovatus* by light microscope. A chorion with more than one layered-chorion has been recorded in the oocyte of some other teleost fishes. The mature oocyte of *Carassius auratus* had a three-layered chorion

(Cotelli *et al.*, 1988). The egg envelope of the full-grown oocyte before fertilization in viviparous species of Goodeidae was composed of one to three layers: filamentous-zona radiata interna and an electron dense zona radiata externa covered by an additional flocculent layer (Riehl and Greven, 1993). Other oviparous teleosts exhibited a division of the zona radiata into two or more layers (Schoots *et al.*, 1982). The chorion thickness of viviparous fishes was considerably reduced in comparison with that of related oviparous species (Riehl and Greven, 1993).

3.3.3 Egg surface

The result of SEM revealed that, the outer surface of the unfertilized egg was smooth and exhibited no projection, whereas the fertilized egg acquired a base like circle network of projection on the vegetal hemisphere, which might represent an adhesive apparatus for contacting substratum. White spots were noticed within the network projections. Such network projections and their related white spots were unique for *C. gariepinus* fertilized egg, since no *Clarias*-related studies referred to such structures. Moreover, no chorion-related studies on other teleost fishes referred to such projections. Different patterns of ornamentation including debris-like dots and batches, irregularly lobulated ornamentations and partially reticulated debris were recorded during the current work on different regions of the egg surface and at different developmental stages. The debris-like dots and the partially reticulated debris might represent the poorly preserved remains of the diffuse mucus layer (Lonning and Hagstrom, 1975; Johnson and Werner, 1986). Similar ornamentation patterns have been recorded by many authors working on different teleost species belonging to different taxonomic groups (Johnson and Werner, 1986; Cotelli *et al.*, 1988; Merrett and Barnes, 1996; Chen *et al.*, 1999; Rizzo *et al.*, 2002; Chiou *et al.*, 2004). Some of these authors considered the egg surface ornamentation as taxonomic characters at the specific level. The variability in ornamentation of *C. gariepinus* makes their use in species identification difficult owing to their association with the fertilization and development process. Chen *et al.* (1999) referred to the importance of the outer surface of the chorion in egg identification and phylogenetic studies. However, they added that the outer surface of the chorion did not show remarkable differences in microstructure among species in a genus or a family.

3.3.4 The micropyle

The structure of the micropyle of *C. gariepinus* egg was similar to the Type III micropyle described by Riehl and Schulte (1977) inasmuch as no micropyle pit was recorded in *C. gariepinus* egg. No micropylar pit was found in *Epinephelus malabaricus*, *E. coioides*, *Sciaenops ocellatus*, and *Mugil cephalus* (Li *et al.*, 2000). The narrowing of the micropyle which recorded in the present work is involved in the polyspermy-preventing reaction (Hart, 1990; Linhart and Kudo, 1997). Riehl and Schulte (1977) described two other types of micropyle; a micropyle with a deep pit and short micropylar canal (Type I) and a micropyle with a flat pit and a corresponding longer canal (Type II). In addition, a cylindrical, a conical and a funnel shaped micropyle have been described in *Gadus morhua marisabli*, *Mugil cephalus* and *Gleginus navaga* respectively (Mekodina, 1987).

Deung *et al.* (1997; 1999; 2000), Kim (1998) and Kim *et al.* (1993; 1996; 1998a, b; 2001; 2002) described the micropyle of different species belonging to four families: Cichlidae, Characidae, Cyprinidae and Belontiidae referring to the taxonomic validity of the micropyle (the egg built-in advantage; Brummett and Dumont, 1979). The micropyle represents the initial isolating mechanism for preventing hybridization (Chen *et al.*, 1999) at least in teleost species since it is considered to be species-specific (Kobayashi and Yamamoto, 1981).

A fur cap micropylar disc was formed with developmental progress in *C. gariepinus* egg. Riehl and Appelbaum (1991) and Wenbiao *et al.* (1991) referred to this micropylar disc. The development of such a micropylar disc made *C. gariepinus* eggs have a unique characteristic shape that differ from that of other catfish such as *Silurus glanis* (Kobayakawa, 1985; Riehl and Appelbaum, 1991). The micropylar disc of *C. gariepinus* resembled that of *P. mattereri*, *S. spiloplema*, *R. aspera*, *Cichlasoma nigrofasciatum* and *Polypterus* spp. (Wirz-Hlavacek and Riehl, 1990; Riehl and Appelbaum, 1991; Bartsch and Britz, 1997; Rizzo *et al.*, 2002). In the absence of data that elucidate the mechanism by which eggs of these species adhere to the substratum, Riehl and Appelbaum (1991) concluded that the micropylar disc may play a role in the attachment process. Similarly, Wenbiao *et al.* (1991) termed the micropylar disc the attachment disc. In such a case, the eggs must be fertilized before their attachment to the substratum (Wirz-Hlavacek and Riehl, 1990). Most catfish including *C. gariepinus* possess demersal eggs which become sticky after contact with water (Riehl and

Appelbaum, 1991). Catfish eggs adhere to the substratum with several other methods (Riehl and Appelbaum, 1991). In *Silurus glanis* and two Japanese *Silurus* species (*S. asotus* and *S. biwaensis*), the eggs adhered via a voluminous layer of jelly (Kobayakawa, 1985; Hilge *et al.*, 1987; Riehl and Appelbaum, 1991) whereas the eggs of the Japanese *Silurus lithophilus* were not adhesive. The jelly layer coat was also present in the adhesive egg of other siluriformes including *Ictalurus* spp. and *Chrysichthys* spp. in addition to *Silurus* spp. (Sato, 1999; Rizzo *et al.*, 2002) and in non-adhesive eggs of siluriformes including *Paulicea luetkeni*, *Pimelodus maculatus* and *Conorhynchus conirostris* (Sato, 1999; Rizzo *et al.*, 2002). The adhesive apparatus of *C. gariepinus* consisted of a large number of tiny attaching filaments, which were embedded in a cement substance (Riehl and Appelbaum, 1991). Such tiny structures were observed in the present work as microvilli extending from the outer surface of the chorion in embryonic stages before hatching.

Linhart and Kudo (1997) reported that the multiple micropyles recorded in some fishes such as Acipenserid species, are less favorable to the prevention of polyspermy than fish eggs possessing only one micropyle. They also referred to the uncertainty of how the multi-micropylar eggs responded to the stimulus of the fertilization by forming a cytoplasmic process underneath several micropyles of the same egg by polyspermy or other mechanisms. Brummett and Dumont (1979) stated that the main block to polyspermy in teleosts was inherent to the morphology of the chorion with its single point of entry. Cosson *et al.* (2002) reported that for spermatozoa the interface trapping mechanism was a very efficient means of increasing their concentration on a surface of the egg instead of their being dispersed in a three-dimensional volume. They also added that this mechanism was highly efficient and crucial for species in which spermatozoa had a very short period of motility to reach the micropyle. Our findings were also corroborated by the observation of the experimentally induced polyspermic eggs by Iwamatsu and Ohta (1978) and Ohta (1985), who reported that spermatozoa can bend and enter the teleost egg at locations other than the micropyl. Moreover, in their studies of polyspermic and monospermic eggs of *Oryzias latipes*, Iwamatsu and Ohta (1978; 1981) described folds of the egg surface that rapidly engulf the sperm in a “Cave-like pit” before the fusion of egg and sperm plasma lemma, which occurred some 20 s later. The sperm behavior of *C. gariepinus* on the chorion surface, micropylar area, micropyle-like depression and folded chorion indicate that the spermatozoa are oriented

inherently toward any depression on the chorion surface in addition to the trapping mechanism of the chorion fibers.

3.3.5 The chorion structure and fertilization

The chorion of unfertilized egg of *C. gariepinus* consisted of one layer. Directly after fertilization, the single layered chorion was differentiated into zona radiata externa (ZRE) and zona radiata interna (ZRI). One hour after fertilization, the chorion was differentiated into three layers double-layered coat (DLC), ZRE and ZRI. The disappearance of the ZRE and the increased tubular nature of the ZRI in the vegetal pole facilitated the embedding of spermatozoa on this region. Griffin *et al.* (1996) reported for *C. pallasi* that the chorion at the animal pole was functionally, structurally and biochemically distinct from the remainder of the surface. The postfertilization changes of the chorion of *C. gariepinus* reflected the morphological aspect of the chorion hardening and fertilization processes. Variations in chorion thickness and rigidity with development and two to four layers, ZRE and ZRE are common among fishes (Kim *et al.*, 1993; Riehl and Greven, 1993; Kim *et al.*, 1996; Deung *et al.*, 1997; Kim, 1998; Kim *et al.*, 1998a; b; Deung *et al.*, 1999; 2000; Kim *et al.*, 2001; Kim *et al.*, 2002).

Based principally on studies with eggs of echinoderms (Vacquier, 1981), the cortical layer of the egg was considered to be a gel with specialized viscoelastic mechanical properties (Hart, 1990). Moreover, the egg cortex became increasingly contractile after sperm-egg union causing twisting movement of oil droplets toward the animal pole; a meshwork of polymerized actin appeared in the egg cytoplasm and microfilaments became highly organized in the microvilli (Vacquier, 1981; Mabuchi, 1983). Actin and actin-containing filaments have been described in the cortical layer of *Brachydanio rerio* (Wolenski and Hart, 1988) and *Oncorhynchus mykiss* (Kobayashi, 1985) eggs. These lectins, as major components in vertebrate cortical granules (Krajhanzl, 1990), are involved in the formation of the egg envelope and intern in their polyspermy-blocking functions (Quill and Hedrick, 1996). Dong *et al.* (2004) identified a C-type lectin from oocytes of a freshwater fish species *Carassius auratus gibelio*. The determination of the chemical and molecular composition of cortical granules is essential to understanding the role of these organelles in fertilization and early development (Hart, 1990). Cortical reaction seems to be a prerequisite for the chorion hardening process which has been considered by some authors to be independent of fertilization (Lonning *et al.*, 1984; Davenport *et al.*, 1986). There was a precise

relationship between sperm behavior, chorion hardening and cortical reaction of *C. gariepinus*.

3.3.6 Chorion proteins and their change during the hardening process

The chorion hardening is a process of initiated chain polymerization of substances within the membrane itself (Hart, 1990) to form insoluble proteins of higher molecular weight (Yamagami *et al.*, 1992). The solubility of the chorion of unfertilized eggs is a requirement for the hardening system or machinery incorporated in the egg envelope (Quagio-Grassiotto and Guimaraes, 2003). The variability in the electrophoretic patterns reported for teleost chorion might be explained by species difference (Griffin *et al.*, 1996). Also, the variability might be due to the difficulty of extracting certain polypeptides from the teleost chorion (Oppenberntsen *et al.*, 1990). The chemical analysis of chorion protein subunits of *C. gariepinus* from 2 min to 3 min after fertilization by partial hydrolysis indicated the raw material of the polymerization process. Zotin (1958) indicated that the chorion began to harden from between <1 min of insemination to 2 to 4 h in salmonid and coregonid eggs, whereas Hart (1990) found that maximum hardening of the chorion was reached within 3-7 days in trout and 1-2 days in whitefish. For the lump sucker (*Cyclopterus* species) and the cod (*Gadus morhua*) chorion hardening started shortly after exposure to sea water and reached a maximum resistance by about 24 h (Lonning *et al.*, 1984). Hardening in these two salt water species did not require fertilization (Hart, 1990).

CHAPTER 4

Embryonic development

4.1 Introduction

Studies on the embryonic development of fishes are important not only to increase the knowledge about the developmental processes but also to understand species-specific adaptations and their ecological value in the course of speciation. Also, the economic relevance for aquaculture and fisheries biology is generally accepted (Meijide and Guerrero, 2000). In spite of the short developmental time, the ease of culturing and a year round reproduction, studies on the embryonic and larval development of *C. gariepinus* are rare comprising those of Bruton (1979), Zaki and Abdula (1983) and Herath (1988) in addition to some brief reports on the spawning and development of this species in the wild (De Graaf and Janssen, 1996).

Still, knowledge of early ontogeny is of critical importance in understanding the biology of a species and the functional trends and environmental preferences of the different developmental stages (Koumoundouros *et al.*, 2001; Borcato *et al.*, 2004). A detailed understanding of the ontogeny is therefore essential to identify species-specific adaptations and their ecological consequences (Verreth *et al.*, 1992). There is no detailed analysis of the anatomical ontogeny of *C. gariepinus*, except for the papers of Stroband and Kroon (1981) and Verreth *et al.* (1992) who studied the organogenesis of the stomach and the digestive system. There is no literature describing the ontogenetic development of the sense organs, skeletal system and circulatory system. So the present work was undertaken to fill some of these gaps and to improve the knowledge about the developmental biology of *C. gariepinus*. We describe the early developmental stages from fertilization to the end of the endogenous feeding period and follow the anatomical ontogeny of selected organs (digestive system, notochord, eyes, ear, and heart).

4.2 Results

4.2.1 Embryonic development - egg stages

The size of the unfertilized eggs of the African catfish *C. gariepinus* was 1.3-1.6 mm in diameter. The stripped eggs are more or less transparent, flattened and brownish

green in color. The egg membrane was separated from the rest of the egg by a small Perivitelline space (Fig. 14a). Directly after insemination the eggs began to swell leading to an increase in egg diameter. One min post-fertilization the adhesiveness of the egg membrane became apparent and the eggs adhered to the substratum.

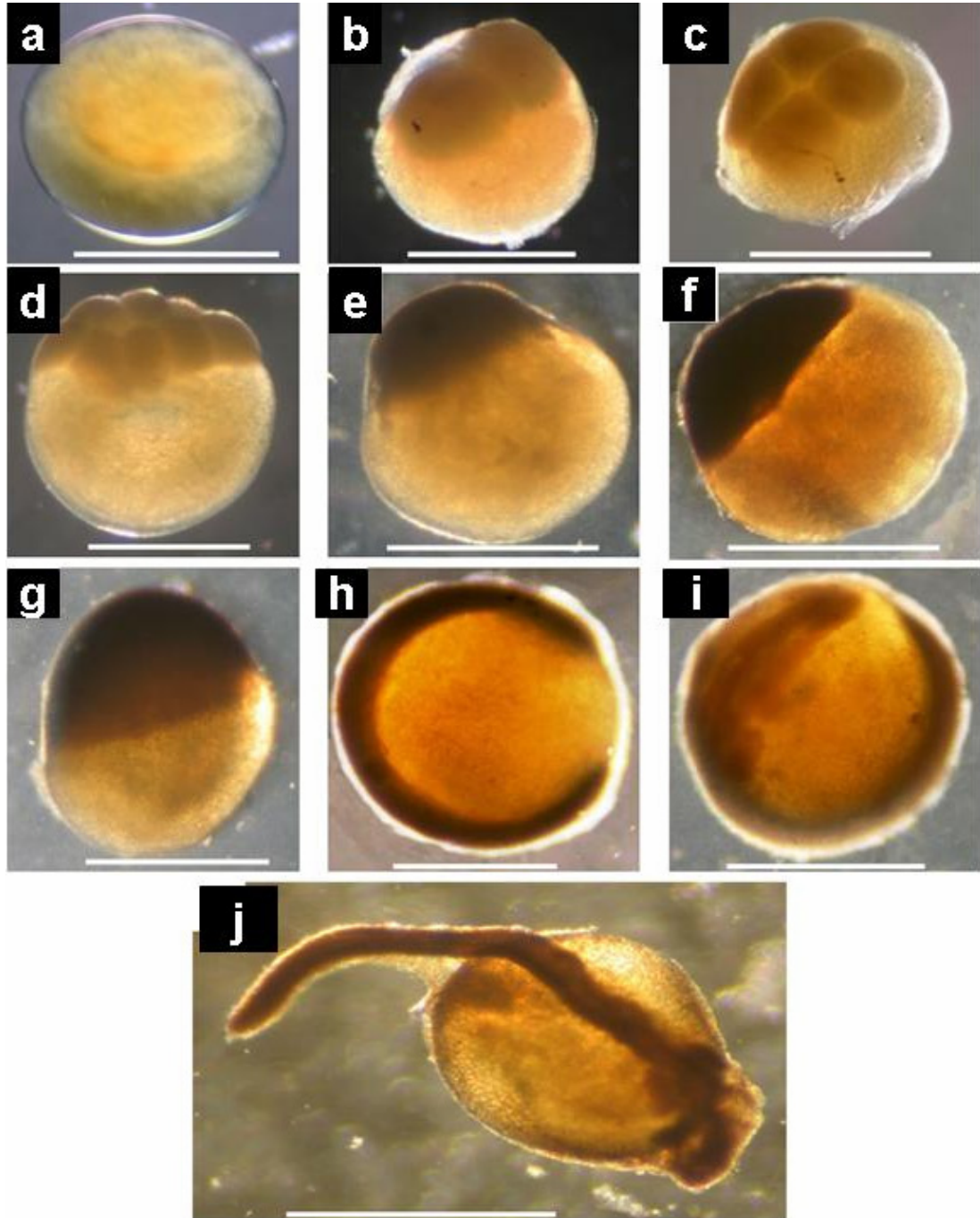


Fig. 14. Egg fixed stages of *Clarias gariepinus* showing (a) unfertilized egg, (b) 2-blastomere stage (50 min post-fertilization), (c) 4-blastomeres stage (70 min post-fertilization), (d) 8-blastomere stage (100 min post-fertilization), (e) morula (4 h post-fertilization), (f) blastula (6 h post-fertilization), (g) gastrula (12 h post-fertilization), (h) C-shaped embryo (24 h post-fertilization), (i) C-shaped embryo with neural tube (about 30 h post-fertilization), (j) embryo dissected of the chorion (36 h post-fertilization). Scale bare = 1 mm.

4.2.1.1 Cleavage phase:

First cleavage occurred at the animal pole (discoidal cleavage) 50 min post-fertilization forming two equally sized blastomeres (Fig. 14b). The 4- and 8-blastomere stages appeared 70 and 100 min post-fertilization (Fig. 14c, d). The 16-blastomere stage was observed at 120 min post-fertilization, followed by the 32-cell stage at 135 min post-fertilization. In the latter stages two cyncytial layers were recognized. A morula formed 4 h post-fertilization (Fig.14e). At the morula stage, cleavage events were non-synchronized.

4.2.1.2 Blastula:

At 6 h post-fertilization flattening of the cellular material occurred, leading to the formation of the blastula (Fig.14f).

4.2.1.3 Gastrula:

Cells of the disc spread over the yolk mass towards the vegetal pole replacing the blastoderm margin and initiating gastrulation. A circular furrow on the underside of the germ disc indicated the germ ring. At 9 h post-fertilization epiboly covered nearly half of the yolk (50 % epiboly stage) (Fig.14g). 12 h post-fertilization the process of epiboly was completed and the embryonic shield was formed.

4.2.1.4 Organogenesis:

The process of organogenesis started when first somites differentiated. At 20 h post-fertilization the embryo encircle the yolk mass. 24 h post-fertilization the embryo was C-shaped (Fig.14h) and organogenesis of the notochord and the heart was initiated. 30 h post-fertilization the future brain was started to differentiate (Fig. 14i). Few hours later, a weak movement inside the transparent eggs was observed. Fig.14j represents an embryo dissected from the chorion 36 h post-fertilization. The head was small and strongly curved on the yolk sac, partitions of the brain were observed, and the beating of the tubular heart was noticed. The tail was not attached to the yolk-sac.

4.2.1.5 Hatching:

The embryos showed twisting movement inside the eggs few hours before hatching. Forty hours after fertilization the egg membrane was ruptured with the caudal region of the embryo and the tail emerged followed by the rest of the body.

4.2.2 Embryonic development - eleutheron-embryonal stages

4.2.2.1 Newly hatched embryo:

The body of the newly hatched embryo remained in a curved position for few hours after hatching, with the head bend down over the yolk (Fig. 15a). The length of the embryo was 4.6 mm and the number of myotomes was ≥ 50 . The newly hatched embryo was transparent, light yellowish in color and lacking pigmentation. Mouth and anus were not opened yet and the eyes were still translucent. A thin membranous fin fold surrounded the caudal region (Fig. 15b).

4.2.2.2 24 hour post-hatching stage (24h-PHS):

At 24h-PHS the embryo measured about 5.2 mm. The number of myotomes increased to 67, the size of the yolk sac was reduced and the membranous fin fold expanded towards the anterior region (Fig. 15c). The tail was curved and the barbells appeared as tiny knobs (Fig. 16a). The mouth and the eyes were still unidentifiable (Fig. 16a). The alimentary canal could be seen as a straight tube emerging from the post-dorsal part of the yolk sac (Fig. 15c). The anal opening was still closed.

4.2.2.3 48h-PHS:

The length of the embryo at this stage was 5.4 mm. The membranous fin fold surrounded the entire area from behind the head region (Fig. 15d). Optic and auditory vesicles could be distinguished (Fig. 16b). The yolk sac was reduced and changed into conical shape. The mouth and the jaws began to differentiate and the barbels became elongated and prominent around the mouth (Fig. 16b). The mouth was still in ventral position constantly moving toward its final terminal position. The anus had not yet formed (Fig. 15d).

4.2.2.4 72-PHS:

72 h-PHS the embryo measured 6.0 mm in length (Fig. 15e). The yolk sac was reduced. The barbells became longer around a well developed mouth. The eyes further differentiated and pigmentation appeared around the head and snout (Fig. 16c). The mouth reached an intermediate position and the operculum could be distinguished (Fig. 16c).

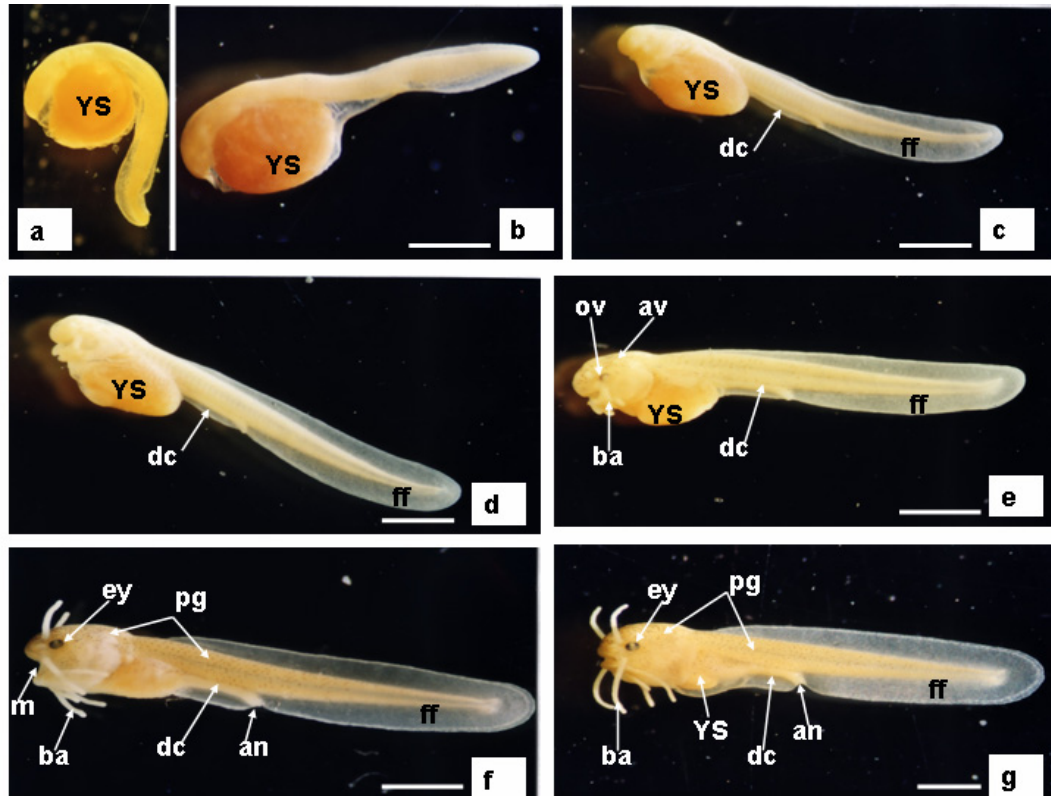


Fig. 15. Eleutheron-embryonal fixed stages of *Clarias gariepinus* showing (a) newly hatched embryo, (b) 4h-PHS, (c) 24h-PHS, (d) 48h-PHS, (e) 72h-PHS, (f) 96h-PHS, (g) 120h-PHS. ys = yolk sac, ff = fin fold, dc = digestive canal, av = auditory vesicle, op = optic vesicle, ba = barbells, m = mouth, ey = eye, pg = pigments, an = anus, PHS = post hatching stage. Scale bare = 1 mm.

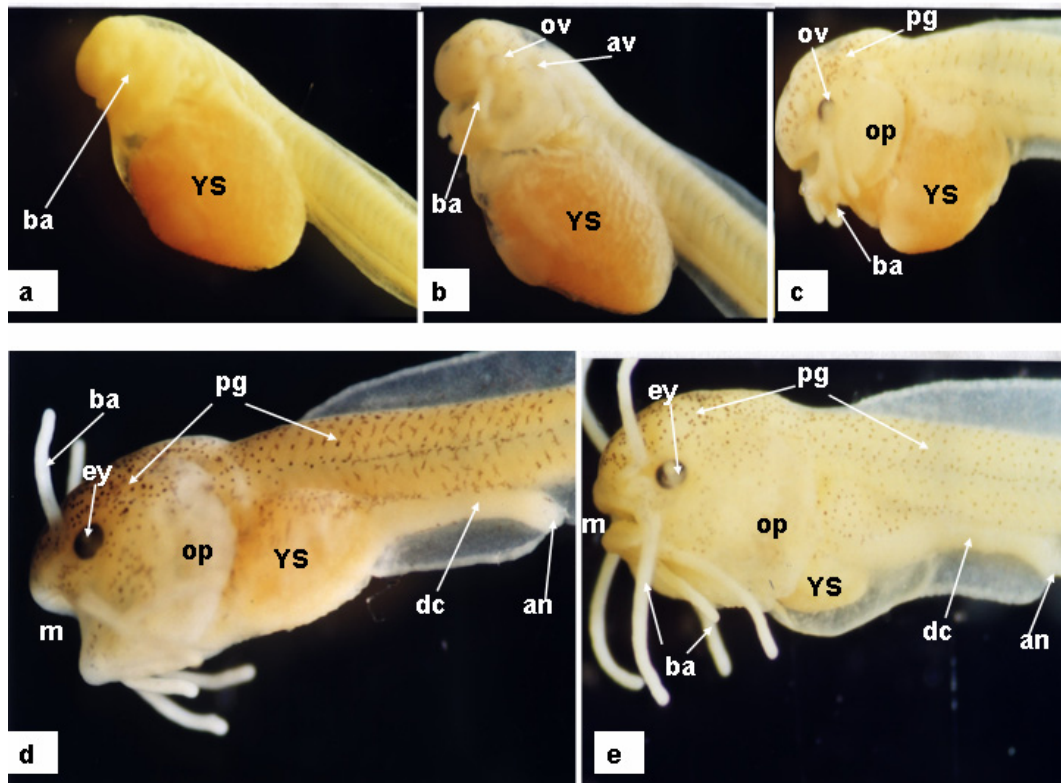


Fig. 16. The development of the head region of *Clarias gariepinus* during eleutheron-embryonal fixed stages. (a) 24h-PHS, (b) 48h-PHS, (c) 72h-PHS, (d) 96h-PHS, (e) 120h-PHS. ys = yolk sac, op = operculum, dc = digestive canal, av = auditory vesicle, op = optic vesicle, ba = barbells, m = mouth, ey = eye, pg = pigments, an = anus, PHS = post hatching stage.

4.2.2.5 96-PHS:

At this stage embryos were 6.2 mm long. The yolk sac was resorbed nearly completely (Fig. 15f). The mouth and the eyes were well developed and the eyes fully pigmented (Fig. 16d). Four pairs of barbels got their final shape. The mouth was in terminal position (Fig. 16d). The pigmentation increased on the head and snout and extended over the whole body except the caudal region (Fig. 16d). The operculum became wider and the anus seemed to be functional (Fig. 16d).

4.2.2.6 120h-PHS:

At 120h post-hatching the length of the embryo was 7.2 mm. The remains of the yolk sac were gradually replaced by the developing alimentary canal until the yolk sac was completely resorbed (Fig. 15g). The operculum and the eyes were fully developed (Fig. 16e). The melanophores became denser including the caudal region (Fig. 15g).

4.2.3 Ontogenetic development of tissues and organs

During organogenesis assignment to distinct stages was not feasible and the course of development is described for each organ individually.

4.2.3.1 Notochord and somites:

First morphological structures of the notochord and somites became visible within the embryonic shield 30 h post-fertilization. 17 somites were formed along the body axis adjacent to the notochord (Fig. 17a). At hatching, the notochord consisted of two types of cells, the peripheral ones forming the notochordal epithelium and the vacuolated cells which represented the largest portion of the medulla of the notochord. The two types of cells were enclosed in a fibrous sheath (Fig. 17b). The notochordal epithelium was composed of cubical cells with large spherical basal nuclei (Fig. 17b, c). The vacuolated cells had differently sized vacuoles and their nuclei were oval and in peripheral position (Fig. 17b). At 24h-PHS, the number of vacuolated cells increased, occupying the whole core of the notochord (Fig. 17d), followed by a transformation in the notochordal epithelium from cubical to squamous cells (Fig. 17e). During the subsequent stages the size of the notochord and the number of vacuolated cells increased (Fig. 17f, h, j) and the internal notochordal epithelium transformed into a typical squamous epithelium (Fig. 17g, i, k).

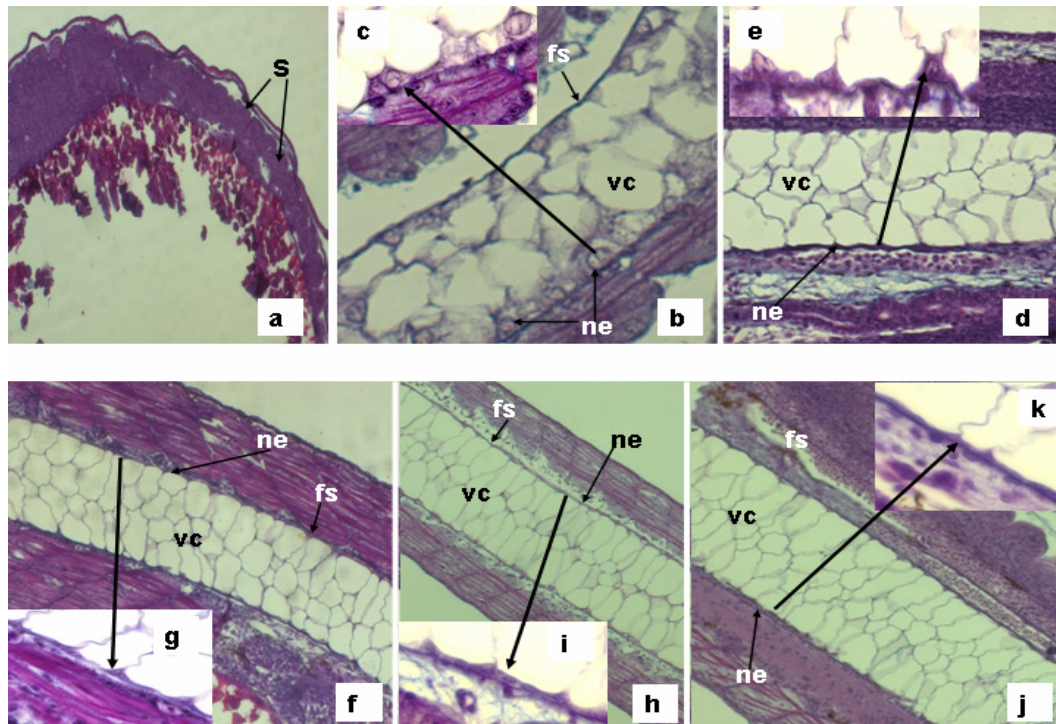


Fig. 17. Sagittal sections through the somites and the notochord of *Clarias gariepinus* showing (a) 30 h post-fertilization stage with 17 somites (50x), (b) notochord of newly hatched embryo (200x), (d) notochord of 24h-PHS (200x), (f) notochord of 48h-PHS (200x), (h) notochord of 96h-PHS (200x), (j) notochord of 120h-PHS (200x), (c, e, g, i, k: x630) showing the transition of notochordal epithelium from cubical (c) into typical squamous epithelium (k). Staining: H&E and Alcian blue. s = somites, fs = fibrous sheath, vc = vacuolated cells, ne = notochordal epithelium, PHS= post hatching stage.

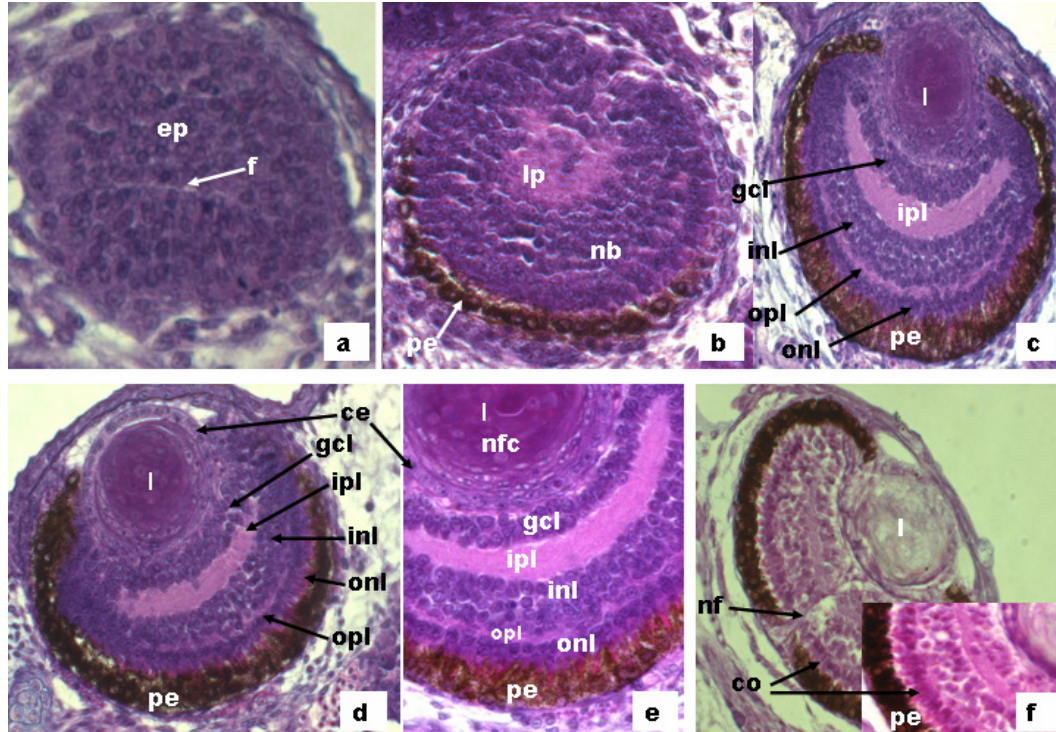


Fig. 18. Sagittal sections through the eyes of *Clarias gariepinus* showing (a) eye of newly hatched embryo (x200), (b) eye of 24h-PHS (x400), (c) eye of 48h-PHS (x400), (d,e) eye of 96h-PHS (x400, x630), (f) eye of 120h-PHS (x400). Staining: H&E and Alcian blue. ep = eye placoid, f = fissure, nb = neuroblastic layer, pe = pigmented epithelium, lp = lens primordial, onl = outer nuclear layer, inl = inner nuclear layer, opl = outer plexiform layer, ipl = inner plexiform layer, gcl = ganglion cell layer, l = lens, ce = cubical epithelium, nfc = nucleated fibrous cells, co = cons, nf = nerve fibres, PHS= post hatching stage.

Indicate age of embryos 120h-PHS the surrounding fibrous sheath thickened and was completely internally lined by a single layer of squamous notochordal epithelium (Fig. 17j, k).

4.2.3.2 Eye:

In the newly hatched embryo the eye placoid was fully differentiated, but not pigmented (Fig. 18a). A small fissure in the center of the eye was formed and might already represent the optic lumen. At 24h-PHS, the neuroblastic layer was differentiated (Fig. 18b), but the cells of the different layers could not be distinguished morphologically. A central primordial lens was apparent. An increasing number of cells containing dark-staining nuclei (melanated pigment epithelium) formed the outer layer of the eye (Fig. 18b). At 48h-PHS the crystalline lens differentiated and the retinal lamination was observed (Fig. 18c). Most of the retina layers (pigmented epithelium, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, and ganglion cell layer) could be recognized and the amount of melanin granules in the pigmented epithelium increased further during this stage. The pigmented layer was clearly developed reaching the lens (Fig. 18c), the outer nuclear layer consisted of a single row of rounded cells, followed by a thin outer plexiform layer. The inner nuclear layer contained three or four rows of spherical cells and a thicker inner plexiform layer was observed (Fig. 18c-e). Both the outer and the inner plexiform layers were eosinophilic. The cells of the ganglion cell layer were uniform in shape, rounded and clearly differentiated. At 96h-PHS (Fig. 18d-e), the deposition of the pigment granules in the pigmented epithelium and the retinal lamination increased further. The crystalline lens was a spherical structure consisting of two types of tissue, the outer encapsulated sheath was composed of cubical cells and the inner one consisting of nucleated fibre cells (Fig. 18e). At 120h-PHS the pigment epithelium was clearly distinguishable as a single layer of cubical cells and the cells in outer nuclear layer started to differentiate into cones. The ganglion cell layers were composed of two layers of spherical cells. The nerve fibres, leading to the optic nerve, were visible on the surface of the retina (Fig. 18f).

4.2.3.3 Inner ear:

At hatching stage, the otic vesicle was oval-shaped and otoliths (lapili and sagitta) were present. The sensory epithelium was situated ventrally, comprising

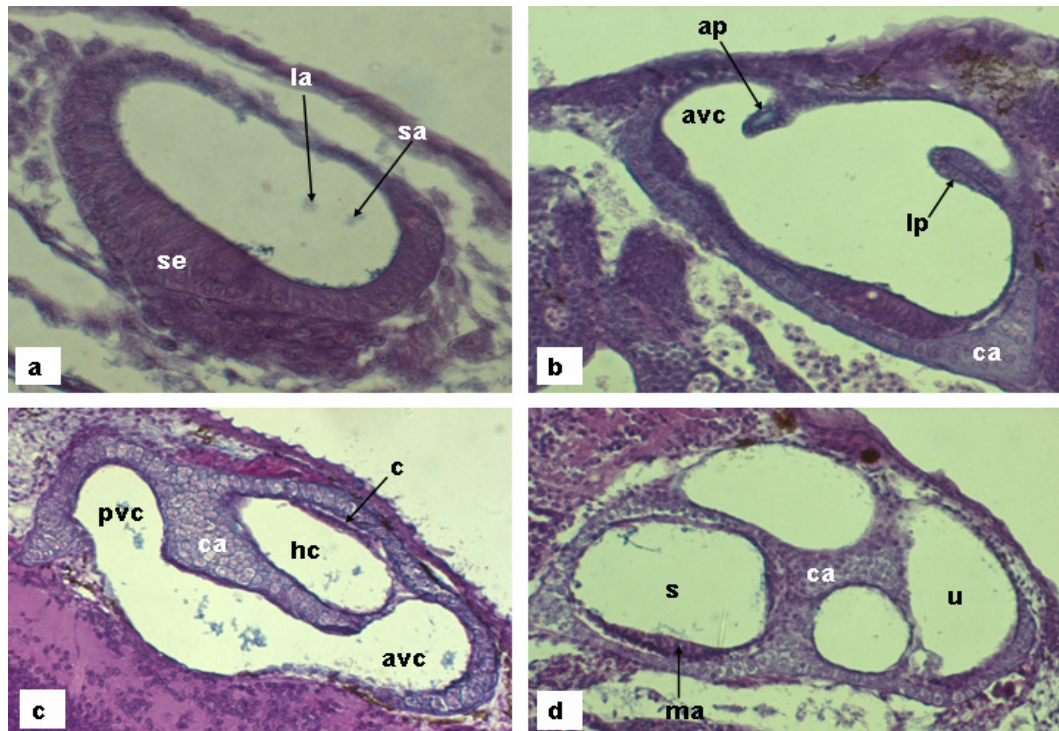


Fig. 19. Sagittal sections through the inner ear of *Clarias gariepinus*, showing (a) inner ear of the newly hatched embryo (x200), (b) inner ear of 24h-PHS (x200), (c) inner ear of 48h-PHS (x200), (d) inner ear of 96h-PHS (x200). Staining: H&E and Alcian blue. se = sensory epithelium, la = lapillus, sa = sagitta, up = upper protrusion, lp = lateral protrusion, avc = anterior vertical canal, pvc = posterior vertical canal, hc = horizontal canal, c = crista, ca = cartilage, u = utricle, s = saccule, ma = macula, PHS= post hatching stage.

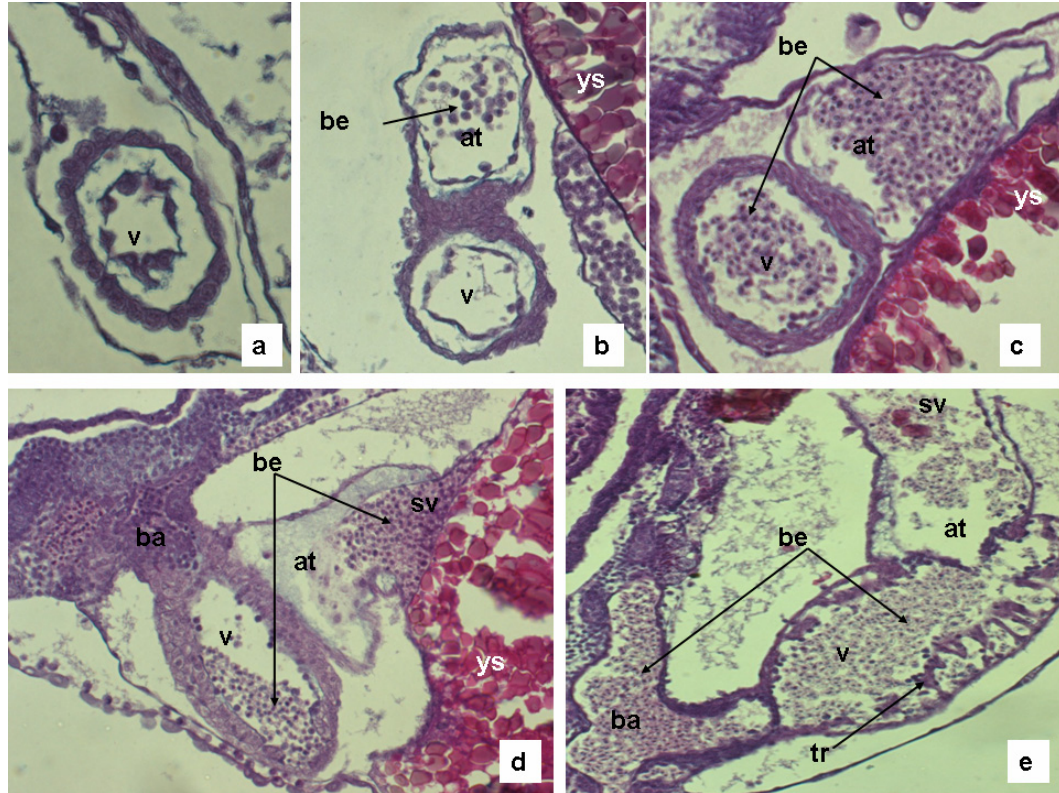


Fig. 20. Sagittal sections through the heart of *Clarias gariepinus* showing (a) heart of the newly hatched embryo (x200), (b) heart of 24h-PHS (x200), (c) heart of 48h-PHS (x200), (d) heart of 96h-PHS (x200), (e) heart of 120h-PHS (x200). Staining: H&E and Alcian blue. v = ventricle, at = atrium, be = blood elements, sv = sinus venosus, ba = bulbus arteriosus, tr = trabiculae, ys = yolk sac, PHS= post hatching stage.

rudiments of the utricular and saccular maculae (Fig. 19a). At 24h-PHS, upper and lateral protrusions of the semicircular canals were distinctive (Fig. 19b).

At 48h-PHS the auditory vesicle enlarged further and three semicircular canals (anterior vertical, horizontal and posterior vertical) with rudimentary cristae (Fig. 19c) were formed. The auditory vesicle was developed at 96h-PHS and the utricular and saccular maculae possessed apical sensory hairs (Fig. 19d), indicating the onset of function.

4.2.3.4 Heart:

The heart was distinguishable as early as 30 h post-fertilization. At hatching, it appeared as a thin-walled two chambered-tube in the front of the yolk-sac. This tubular structure was visible as a ring when viewed from the ventral surface (Fig. 20a). At this stage, no blood elements were observed in the heart. The two chambers (ventricle and atrium) were clearly developed 24h-PHS (Fig. 20b). At this stage few blood elements were recorded. At 48h-PHS the atrium ended on the yolk and the ventricle opened into the dorsal aorta (Fig. 20c). During this stage blood elements were recorded in large quantities in the heart chambers (Fig. 20c). 96h-PHS, four distinct compartments (bulbus arteriosus, ventricle, atrium, and sinus venosus) had formed (Fig.20d). At 120h-PHS numbers of trabiculae differentiated in the ventricle and the wall of the bulbus arteriosus thickened (Fig.20e). The amount of blood and the relative number of mature erythrocytes was increasing steadily.

4.2.3.5 Digestive system:

The digestive system in the newly hatched embryo consisted of a straight undifferentiated tube (Fig. 21a), encompassing a narrow uniform lumen, connected dorsally to the yolk sac. The epithelium was formed by a monostratified layer of cubical/columnar epithelium cells (Fig. 21b). Mouth and anus were still closed. Until 24h-PHS the alimentary canal increased only in length. At 24h-PHS, the cells of the liver and the future pancreas were localised between the digestive tract and the yolk sac. 48h-PHS the digestive system differentiated into buccal cavity, oesophagus and intestine (Fig. 21c), but mouth and anus were still closed (Fig. 21e). The buccal cavity was lined with squamous epithelium (Fig. 21g), the oesophagus appeared as a simple columnar epithelium with narrow and short lumen (Fig. 21d) and its connection with the intestine and the buccal cavity was not yet established. The lining of the rest of the alimentary canal consisted of columnar epithelium.

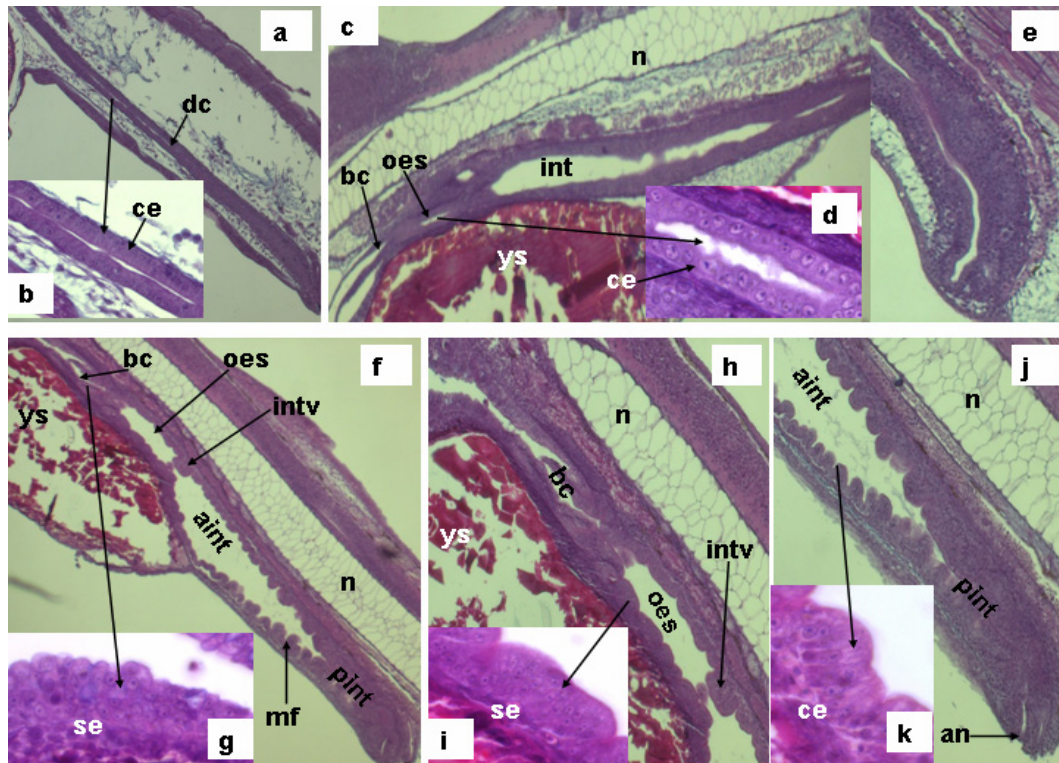


Fig. 21. Sagittal sections through the digestive system of *Clarias gariepinus* showing (a) undifferentiated digestive canal of the newly hatched embryo (x100), (c) digestive system of 24h-PHS (x50), (e) closed anus of 24h-PHS (x100), (f,h) digestive system of 96h-PHS (x50, x100 respectively), (j) the intestine and its opened anus of 96h-PHS (x100), (b,d,k) cubical/columnar epithelium (x630), (g,i) squamous epithelium (x630). Staining: H&E and Alcian blue. bc = buccal cavity, oes = oesophagus, int = intestine, intv = intestinal valve, aint = anterior intestine, pint = posterior intestine, mf = mucosal fold, se = squamous epithelium, n = notochord, ce = cubical/columnar epithelium, ys = yolk sac, PHS = post hatching stage.

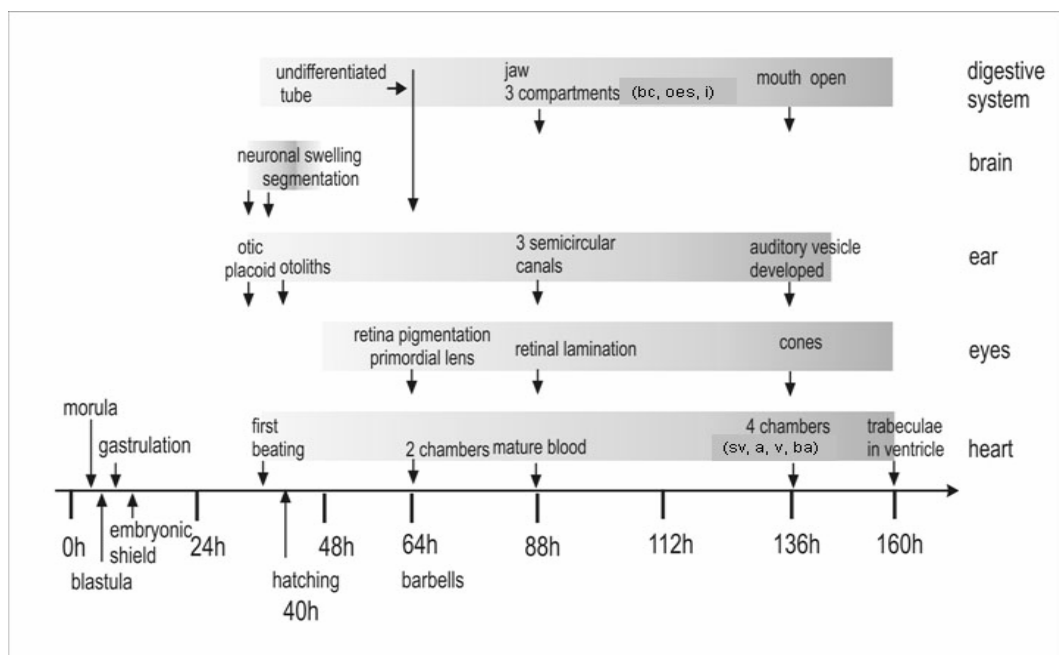


Fig. 22. Embryogenesis in *Clarias gariepinus* focusing on the organogenesis of the heart, notochord, sensory organs and digestive system. bc = buccal cavity, oes = oesophagus, i = intestine, v = ventricle, a = atrium, sv = sinus venosus, ba = bulbus arteriosus

The connection between the oesophagus and the buccal cavity and the intestine was established at 96h-PHS (Fig. 21f). Thereafter, lining of the oesophagus transformed into stratified epithelium (Fig. 21i). An intestinal valve was formed from columnar epithelium between the oesophagus and the intestine (Fig. 21f, h), dividing the digestive tract into three distinct regions: buccal cavity, oesophagus, and intestine. A constriction divided the intestine further into a wide anterior intestine and a narrow posterior intestine (Fig. 21j). This constriction seemed to be an outgrowth of the intestine. Numerous epithelial folds and villi-like structures were observed throughout the intestine (Fig. 21h). At this stage, the mouth and the anus opened.

4.3 Discussion

Despite its economic importance the embryonic and ontogenetic development of *C. gariepinus* is poorly understood and only few studies are available (Bruton, 1979; Zaki and Abdula, 1983; Herath, 1988). Here the entire embryogenesis was described for the first time in *C. gariepinus* with a focus on the organogenesis of the heart, notochord, sensory organs and digestive system (Fig. 22).

High variation in egg size and colouration was recorded for the eggs of *C. gariepinus* (Bruton, 1979; Zaki and Abdula, 1983; Herath, 1988; Verreth *et al.*, 1993). This might be related to the existence of different races in nature (Thakur, 1980). Alternatively, it may also depend on the individual parental investment, moderated by the food availability experienced by the female spawner. The fertilized eggs of *C. gariepinus* became adhesive, similar to those of other catfish species such as *Clarias batrachus*, *Mystus montanus* and *Pangasius sutchi* (Bruton, 1979; Arockiaraj *et al.*, 2003; Islam, 2005), representing an adaptation to prevent drift and provide optimal oxygen supply. At 24 °C the incubation period (the period from fertilization to hatching) lasted 40-48 h. Zaki and Abdula (1983) and Herath (1988) reported shorter incubation periods at higher temperatures. The development is clearly temperature-dependent as in most fishes (De Graaf and Janssen, 1996).

All teleosts show a discoidal meroblastic cleavage pattern, where the large yolk volume restricts cell division to a small area at the animal pole close to the micropyle (Hall *et al.*, 2004). The pattern of cleavage recorded here for *C. gariepinus* did not markedly differ from that in other catfish, e.g. *P. sutchi* (Islam, 2005) and *M. montanus* (Arockiaraj *et al.*, 2003) or most teleosts such as *Oryzias latipes*, *Danio rerio*,

Cichlasoma dimerus and *Gadus morhua* (Kimmel *et al.*, 1995; Meijide and Guerrero, 2000). However, inter- and intra-specific variation exists in the cleavage pattern (Hall *et al.*, 2004). For example, the first horizontal cleavage occurs between 16- and 32-blastomeres in *C. gariepinus* and medaka *O. latipes* (Iwamatsu, 1994), but it is only observed between 32-cell and 64-cell stage in *D. rerio* and in *C. dimerus* (Kimmel *et al.*, 1995; Meijide and Guerrero, 2000) or even earlier, between the 4-cell and 8-cell stage, in the Ice goby *Leucopsarion petersii* (Nakatsuji *et al.*, 1997). Different timing for the morula and blastula stage were reported for catfish species (Islam, 2005), but are still congruent with the observations reported here. The beginning of gastrulation, defined by the onset of involution, resulted from the movement of embryonic cells over the surface of the yolk (epiboly) and was observed at the 50 % epiboly stage similar to the pattern reported in common carp, medaka and cod (Verma, 1971; Iwamatsu, 1994; Hall *et al.*, 2004). In contrast, gastrulation in zebrafish starts after the 50 % epiboly stage (Warga and Kimmel, 1990). Few hours before hatching the African catfish *C. gariepinus* showed twisting movements inside the egg. This hatching behaviour is commonly observed in catfish, e.g. *Heteropneustes fossilis* (Thakur *et al.*, 1974), *C. batrachus* (Thakur, 1980) and *P. sutchi* (Islam, 2005). Generally, previous observations on the early development of *C. gariepinus* (Bruton, 1979; Zaki and Abdula, 1983; Herath, 1988) and also on other catfish species (Arockiaraj *et al.*, 2003; Islam, 2005) agree with the pattern described in the present study, thus confirming the basic uniformity of catfish development particular in pre-hatching stages.

The newly hatched embryos of *C. gariepinus* were characterized by a large yolk sac which they nourished here for 120h-PHS. At 30 °C the yolk sac period lasted only 48 h (Verreth *et al.*, 1992) indicating a high influence of the temperature regime in *C. gariepinus* embryonic development. According to the present observations, the terminal mouth, the jaws, the eyes and the operculum of *C. gariepinus* became well developed and the barbels resumed their normal shape 96h-PHS. Obviously, embryos are highly developed with regard to their principal sensory organs as well as to their foraging capacities (jaws, digestive tract, and mouth) before external feeding are initiated. Functionality is most probably reached 24 h before the complete depletion of the yolk sac. Active feeding might even be initiated as early as 24 h before the complete resorption of the yolk sac. In contrast, active feeding of free embryo is assumed inefficient (Drost *et al.*, 1988a, b; Osse, 1990) due to delayed jaw function. As

suggested by the authors, this is compensated for either by filter-feeding and/or endogenous food supply. Additional support for an early onset of external feeding is the morphologic evidence for an early function of the sensory organs. According to the hypothesis of Kawamura and Washiyama (1989), vision is the most important sense of fish larvae used for feeding and prey detection. In *C. gariepinus*, this is supported by well developed eyes 96h-PHS. However, one has to consider that a functional digestive system is completed only 5 days after the onset of exogenous feeding (Verreth *et al.*, 1992). On the other hand, this might even raise pressure for efficient foraging compensating for insufficient digestion.

The notochord, which forms the median rod of the embryo shortly after gastrulation, serves as the major supportive structure of the embryo until ossification of the skeleton (Hall *et al.*, 2004). In the present work, *C. gariepinus* retained an unconstricted notochord till the end of the experiment (120h-PHS). The unconstricted notochord previously observed in the short nose sturgeon, *Acipenser brevirostrum*, and in the African lungfish, *Protopterus annectens*, (Schmitz, 1998) was also found in *C. gariepinus*. The notochord consisted of two types of cells (vacuolated cells and notochordal epithelium) enclosed in a fibrous sheath. This sheath is important in directing notochord elongation, whereas vacuolization provides the motive force via an increase in size (Garrison and Wytenbach, 1985). Here upon hatching, vacuoles in the vacuolated cells differed considerably in size, indicating that the process of vacuolization was initiated shortly before hatching. The increase in vacuole size and the proliferation of vacuolated cells directed pressure on the notochordal epithelium, transforming cuboidal to typical squamous cells.

The ontogenetic development of the eye in *C. gariepinus* included some characteristics commonly observed in several teleost species, eg. *Perca fluviatilis*, *Anguilla anguilla* and *Gadus morhua* (Gumaa, 1982; Pankhurst, 1984; Rodriguez and Gisbert, 2002; Hall *et al.*, 2004). The lack of eye pigmentation at hatching was reported for many fish species such as Atlantic halibut (Kvenseth *et al.*, 1996) and Walleye pollock (Porter and Theilacker, 1999). Based on the lack of pigmentation at hatching, Blaxter (1968) concluded that the eyes of several fish species are almost certainly non-functional. Vice versa, the presence of small scattered dark patches of pigments in newly hatched embryo is often suggested as indication of functionality (Loew and

Sillman, 1993; Boglione *et al.*, 1997; Rodriguez and Gisbert, 2002). Here, pigmentation was recorded shortly after hatching and the retinal layers could be recognized as early as 2 days post-hatching in comparison to 3 days post-hatching in bluefin tuna (Kawamura *et al.*, 2003) or 5 day post-hatching in the Siberian sturgeon (Rodriguez and Gisbert, 2002). The cells of the ganglion cell layer of *C. gariepinus* consisted of two rows of spherical cells and the differentiation of the ganglion layer indicated a possible connection between the retina and the optic tectum prior to the appearance of the outer segments of photoreceptors as described by Kvenseth *et al.* (1996). Thus the transmission of light-induced impulses to the optic tectum has to be considered here. We observed pigment migration around the eye during the retinal lamination. This kind of pigment migration has been recorded in different fish species (Neave, 1984; Kvenseth *et al.*, 1996). However, Blaxter and Staines (1970) could not detect such a migration of pigments in a number of marine fish. The retina of most teleost embryos is initially dominated by cones; rods usually appear some time after (Blaxter, 1986; Pankhurst and Eagar, 1996). Acute vision in the turbid freshwater relies on cones and is therefore more important in catfish embryos than high visual sensitivity transmitted via rods. Congruently, 120h-PHS the pigmented epithelium consisted of a single layer of cubical cells and an outer nuclear layer composed of cones. In contrast, bluefin larvae develop rods 14 days earlier than the cones (Kawamura *et al.*, 2003), representing an adaptation to larval feeding under dim light (Kawamura *et al.* 1984). Obviously, vision and its species-specific realization during ontogeny reflect the adaptation toward the natural habitat and its ecological constraints.

In teleost the inner ear consists of two functionally discrete regions: the vestibular region (the utricle and three semicircular canals) and auditory region (the saccule and lagena) (Hoss and Blaxter, 1982). Little is known about the histological and /or cytological development of the inner ear in fish (Matsuoka, 2001). Kawamura and Ishida (1985) and Kawamura and Washiyama (1989) reported an oval-shaped inner ear with two otoliths comparable to the observations in this study. The three semicircular canals (anterior vertical, horizontal and posterior vertical) with rudimentary cristae had formed at 48h-PHS providing the ability to establish an upright position and to initiate intentional swimming at this time (Matsuoka, 2001). The sensory epithelium described here might enable the hatched embryos to maintain a 3-D orientation, detect vibration in the water and avoid moving pipet. Recently, similar conclusion were drawn by

Kawamura *et al.* (2003). Therefore, functionality of the inner ear can be assumed shortly after hatching in *C. gariepinus*. A functional acoustic sense preceding other organs for example retinal pigmentation or mouth opening might be essential for predator avoidance, when free embryos are highly vulnerable. In contrast to the variability of eye development in fish, the inner ear development seems similar among species emphasizing the early need for 3-D orientation in larvae.

Development of the heart and vascular system varies substantially among teleosts, often reflecting egg and larval size (Hall *et al.*, 2004). Beating of the heart was observed as early as the 30 h post-fertilization. Hall *et al.* (2004) recorded the first heart beat in Atlantic cod shortly before the 40-somite stage, which makes the heart the first organ to form and function. One day post-hatching few blood elements were recorded in the heart of *C. gariepinus* but functional gills were not found at this stage. However, the purpose of the initial circulation is likely the distribution of yolk substances rather than gas exchange (Hall *et al.*, 2004). Heart development was relatively fast in *C. gariepinus* similar to that of common dentex *Dentex dentex* (Santamaria *et al.*, 2004). Like *C. gariepinus* common dentex is a subtropical fish, suggesting that early formation of the heart in subtropical fish represents an adaptation to increased metabolic rates and the resulting nutrient requirement and oxygen demand under higher temperature. Nevertheless, the reason for the initiation of larval heart beating is still debated controversially. Although the thesis that initiation of pumping reflects the need for convection is widely accepted, other authors (e.g. Burggren, 2004) argue that there is no compelling evidence for this as fish, amphibian and even bird embryos lacking a functional heart (surgical intervention or mutation) can resume development at this stage based on diffusion alone.

CHAPTER 5

Lead induced morphological and histological malformations during embryogenesis

5.1 Introduction

The biotic integrity of an ecological system is reflected by the health of its fauna (Adams *et al.*, 1993) and in aquatic ecosystems, fish are regarded as a valuable indicators of environmental pollution. Heavy metals have been a concern for human health and still represent a problem in some countries (Elnabawi *et al.*, 1987; Elsokkary and Muller, 1990; Abdel-Nasser *et al.*, 1996). Lead accumulates chiefly in the bone, scales, gills, kidney, intestine, liver, muscles and to a lesser extent in the brain (Hodson *et al.*, 1978; Holcombe *et al.*, 1979; Hodson *et al.*, 1982; Allen, 1995; Chan, 1995; Farag *et al.*, 1995; Vigh *et al.*, 1996) and tissue accumulation depends upon exposure concentration and duration (Canli and Kalay, 1998).

Exposure of unfertilized egg was reported to impair fertilization process and exposure of fertilized egg effects early cleavage patterns and hatching time (von Westernhagen, 1988). Generally, larvae display a vast array of gross and minor morphological aberrations after exposure to toxic substances (Devlin and Mottet, 1992; Jezierska *et al.*, 2000; Hallare *et al.*, 2005). Morphological aberrations are not pollutant-specific and may be caused also by natural stresses (Nguyen and Janssen, 2002). Beside the morphological aberrations, histopathology has been used in sublethal tests in ecotoxicology in fish (Ortiz *et al.*, 2001) and can be used to establish water quality criteria (Ortiz *et al.*, 2001). Although, few ecotoxicological studies were carried on embryonic and larval stages of the African catfish *C. gariepinus* (Nguyen *et al.*, 1997; Nguyen *et al.*, 1999; Nguyen and Janssen, 2002; Olaifa *et al.*, 2003), there is no literature concerning the morphological and histopathological abnormalities of the African catfish embryos after lead exposure. So, the current study was undertaken to describe lead-induced morphological malformations and histopathological changes in embryonic stages of *C. gariepinus*. The present work aims to detect the effect of lead nitrate on the development and hatching success and to describe lead-induced malformations during embryogenesis. Attention was paid to gross and minor morphological malformations and histopathological changes at the light microscopic level.

5.2 Results

Lead-induced malformations were observed in embryos of the African catfish *C. gariepinus* in pre-hatching and post-hatching stages after lead exposure. An overall delay in development was reported. Compared to the control embryos (Fig. 15), morphological (Figs. 23-26) and histopathological (Figs. 27-30) anomalies in lead-exposed embryos were documented.

5.2.1 Incubation period and hatching success

In the control hatching started 40 h after fertilization. The ratio of newly hatched embryos / fertilized eggs was 20 % at the beginning of hatching. The total percentage of hatched embryos / fertilized eggs was 75 % at 48 h post fertilization stage (48h-PFS). Embryos exposed to 100 µg/l lead began to hatch at 40 h post-fertilization (20 % of newly hatched embryos / fertilized eggs). The total percentage of hatched embryos / fertilized eggs was 65 % at 48h-PFS. In the groups exposed to 300 µg/l and 500 µg/l developmental time was prolonged and hatching started at 50 h post-fertilization, with a ratio of 5 % newly hatched embryos / fertilized eggs. The hatching rate was 50 % and 40 % for 300 µg/l and 500 µg/l, respectively. The data indicated that exposition to lead nitrate caused a concentration-dependent delay in hatching and also reduced the percentage of embryos which successfully completed the egg stage period.

5.2.2 Morphological aberrations in post-hatching stages

Four major categories of gross morphological abnormalities (irregular head shape, pericardial oedema, yolk sac oedema and notochordal defect) and two minor deformities (finfold defect and reduction of pigmentation) were observed. Some of the affected embryos were recorded with combinations of these abnormalities.

5.2.2.1 Irregular head shape

At hatching embryos with an irregular head shape were observed only in the 500 µg/l lead treatment (Fig. 23a, b). The malformed head bears swelling like protrusion on the lateral (Fig. 23a) or anterior (Fig. 23b) side. The malformation was often associated with a branched tail (Fig. 23a) or a collapsed one (Fig. 23b). These malformations were lethal and embryos died shortly after hatching.

5.2.2.2 Pericardial oedema

An enlargement in the pericardial sac or pericardial oedema (Fig. 23c) was observed only in the newly hatched embryos when exposed to 500 $\mu\text{g/l}$ lead. Malformed embryos only survived for few hours.

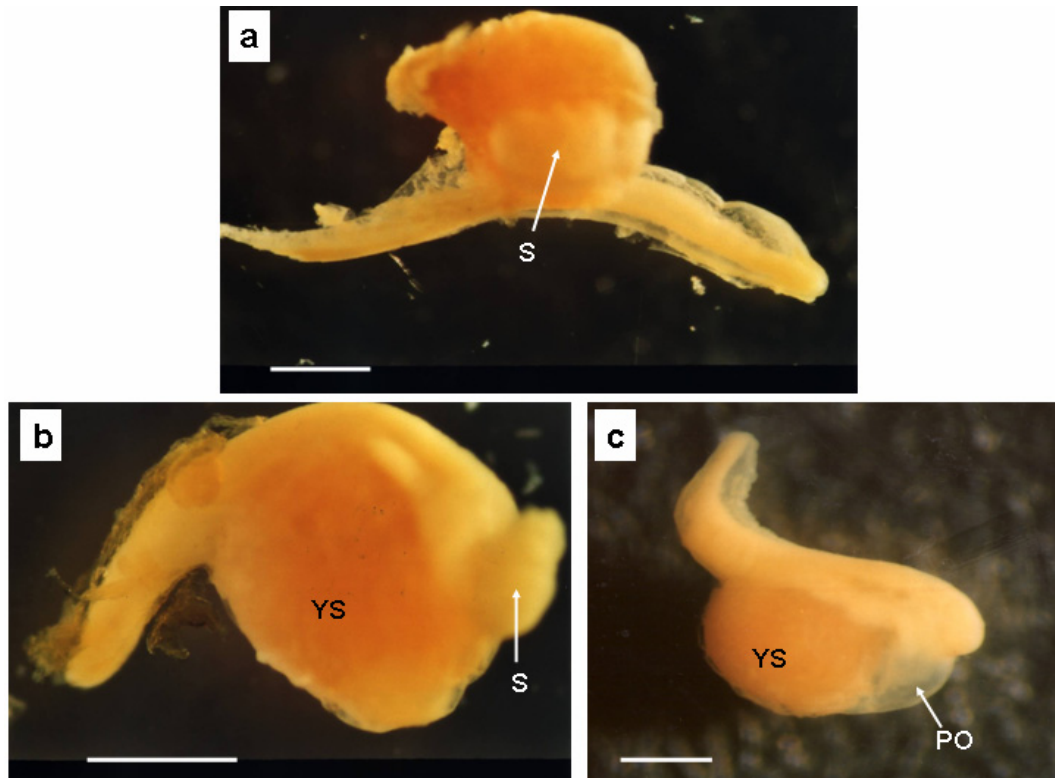


Fig. 23. Deformed newly hatched embryos of *Clarias gariepinus* after exposure to 500 $\mu\text{g/l}$ lead nitrate showing (a) embryo with irregular head shape and branched tail, (b) embryo with irregular head shape and collapsed tail (c) embryo with pericardial oedema. YS= yolk sac, S= swelling, PO= pericardial oedema. Scale bare= 1mm.

5.2.2.3 Yolk sac oedema

Yolk sac oedema was observed at 120h and 144h-PFS (Table 3, Fig. 24a-c) in the groups exposed to 300 and 500 $\mu\text{g/l}$ lead nitrate. Different shapes of yolk sac oedema were observed comprising bag-shape oedema (Fig. 24a), balloon-shape oedema (Fig. 24b) and oval-shape oedema (Fig. 24c). Malformed embryos were characterized by poorly developed mouth and jaws. Also yolk sac oedema was often associated with notochordal (spinal cord) curvature. Oedematous embryos were usually shorter than the normal ones.

5.2.2.4 Notochordal defect

The most frequently observed gross morphological deformation was a bent body or a notochordal (spinal chord) curvature.

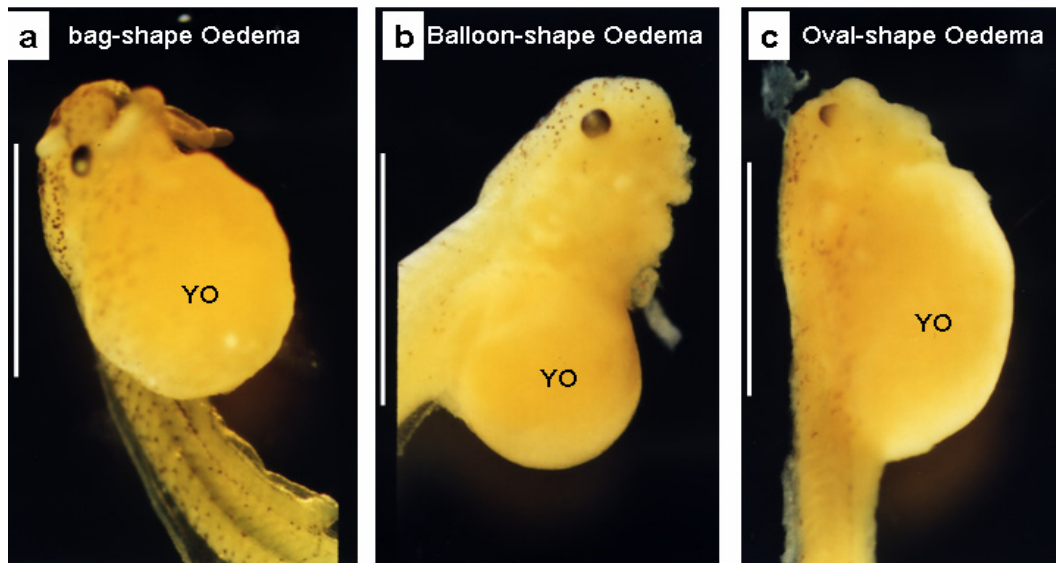


Fig. 24. Yolk sac oedema in the embryos of *Clarias gariepinus* after lead exposure (a) bag-shape oedema (144h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate), (b) Balloon-shape oedema (120h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate), (c) oval-shape oedema (120h-PFS exposed to 300 $\mu\text{g/l}$ lead nitrate). YO=yolk sac oedema, PFS=post fertilization stage. Scale bare= 1mm.

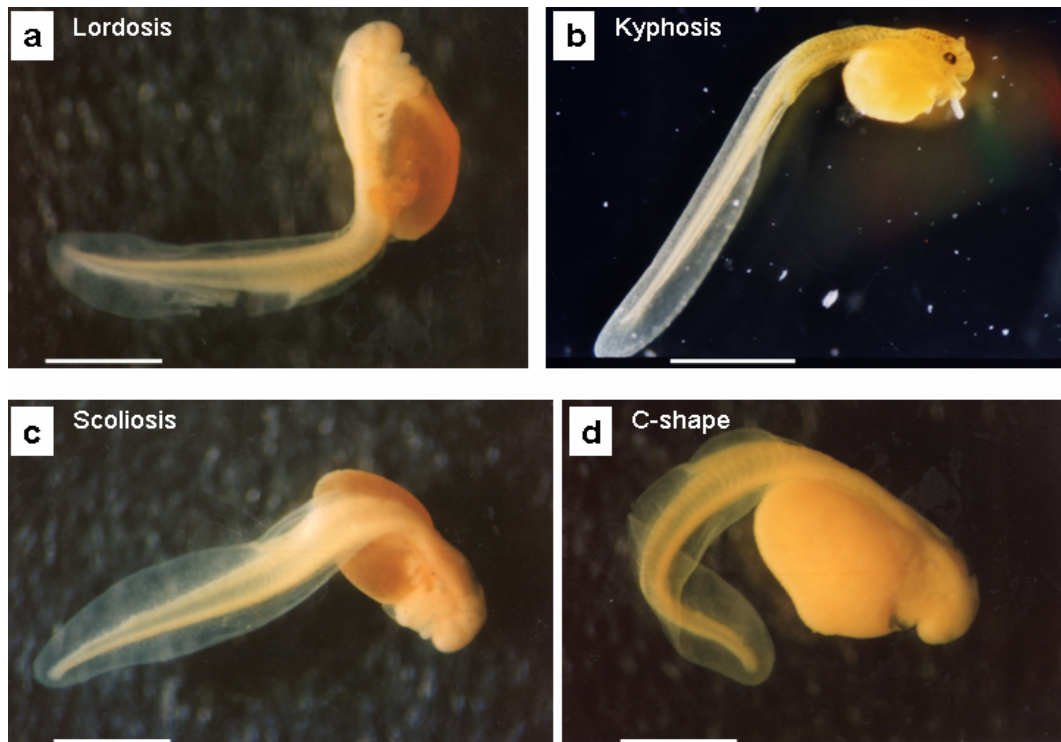


Fig. 25. Notochordal abnormality (body curvature) in the embryos of *Clarias gariepinus* after lead exposure (a) lordosis (96h-PFS exposed to 300 $\mu\text{g/l}$ lead nitrate), (b) kyphosis (120h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate), (c) scoliosis (96h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate), (d) C-shaped body (96h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate). PFS=post fertilization stage. Scale bare= 1mm.

Different types of spinal cord curvature were recorded (Fig. 25a-d) (1) lordosis (dorsoventral curvature) (Fig. 25a), (2) kyphosis (ventrodorsal curvature) (Fig. 25b), (3) scoliosis (lateral curvature) (Fig. 25c), and (4) C-shaped curvature (Fig. 25d). Spinal cord curvature was recorded in all three treated groups (Table 3): At 48h-PFS with 4,3 % frequency in the control group, with 21 % frequency in 100 µg/l treated group, with 36 % frequency in 300 µg/l treated group and with 70 % frequency in 500 µg/l treated group. In the later sampling points, spinal cord curvature was less frequently observed, since the majority of this malformation was lethal (Table 3).

Table 3. Morphological malformations during embryonic development of *Clarias gariepinus* due to the exposure to different concentration of the lead nitrate. Deformations are expressed as percentage of the total number of larvae; means \pm SD (n=3). PFS= Post fertilization stage

Embryonic Stages	Treatments µg/l	Irregular head	Notocordal defect	Yolk sac oedema	Finfold defect
48 h PFS	Control	0,0	4,3 \pm 0,6	0,0	0,0
	100	0,0	21,0 \pm 2,6	0,0	0,0
	300	19.7 \pm 0,6	36.3 \pm 1,2	0,0	0,0
	500	34.3 \pm 1,2	69.7 \pm 0,6	0,0	0,0
72 h PFS	Control	0,0	3,7 \pm 0,6	0,0	1,7 \pm 1,2
	100	0,0	20,7 \pm 1,2	0,0	3,0 \pm 0,0
	300	0,0	33.0 \pm 0,0	0,0	9.7 \pm 3,8
	500	0,0	50 \pm 5,0	0,0	19.3 \pm 0,6
96 h PFS	Control	0,0	2,7 \pm 2,0	0,0	0.7 \pm 1,5
	100	0,0	10.0 \pm 4,0	0,0	4.7 \pm 2,6
	300	0,0	19.3 \pm 2,5	0,0	8.0 \pm 3,6
	500	0,0	22.3 \pm 2,5	0,0	15.0 \pm 0,0
120 h PFS	Control	0,0	0.3 \pm 0,6	0,0	0,0
	100	0,0	4.7 \pm 2,5	0,0	20.3 \pm 4,5
	300	0,0	5.0 \pm 0,0	2.3 \pm 0,6	24.3 \pm 2,1
	500	0,0	12.0 \pm 2,6	3.3 \pm 1,5	35.0 \pm 5,0
144 h PFS	Control	0,0	0,3 \pm 0,6	0,0	0,0
	100	0,0	4,3 \pm 1,5	0,0	17.7 \pm 2,5
	300	0,0	5,0 \pm 0,0	1.0 \pm 1,0	20.7 \pm 5,1
	500	0,0	8,7 \pm 0,6	1.7 \pm 0,6	23.3 \pm 2,9
168 h PFS	Control	0,0	0,3 \pm 0,6	0,0	0,0
	100	0,0	3,7 \pm 1,2	0,0	0,0
	300	0,0	4,3 \pm 0,6	0,0	8.7 \pm 2,5
	500	0,0	7,7 \pm 0,6	0,0	11 \pm 2,0

5.2.2.5 Finfold defects

Blistering/necrosis of fins was one of the dominant minor deformations (Fig. 26a-c). This abnormality was only observed in these exposition groups (100, 300 and 500 $\mu\text{g/l}$). In the group exposed to 100 $\mu\text{g/l}$ the blistering was restricted in the posterior part of the tail (Fig. 26a). While the fin was degenerated through the whole tail in the group exposed to 300 $\mu\text{g/l}$ (Fig. 26b). The membranous fin was completely blistered and degenerated in the groups exposed to 500 $\mu\text{g/l}$ (Fig. 26c). The frequency of larvae with finfold defect increased with lead concentration (Table. 3) and was often associated with yolk sac oedema and/or notochordal curvature.

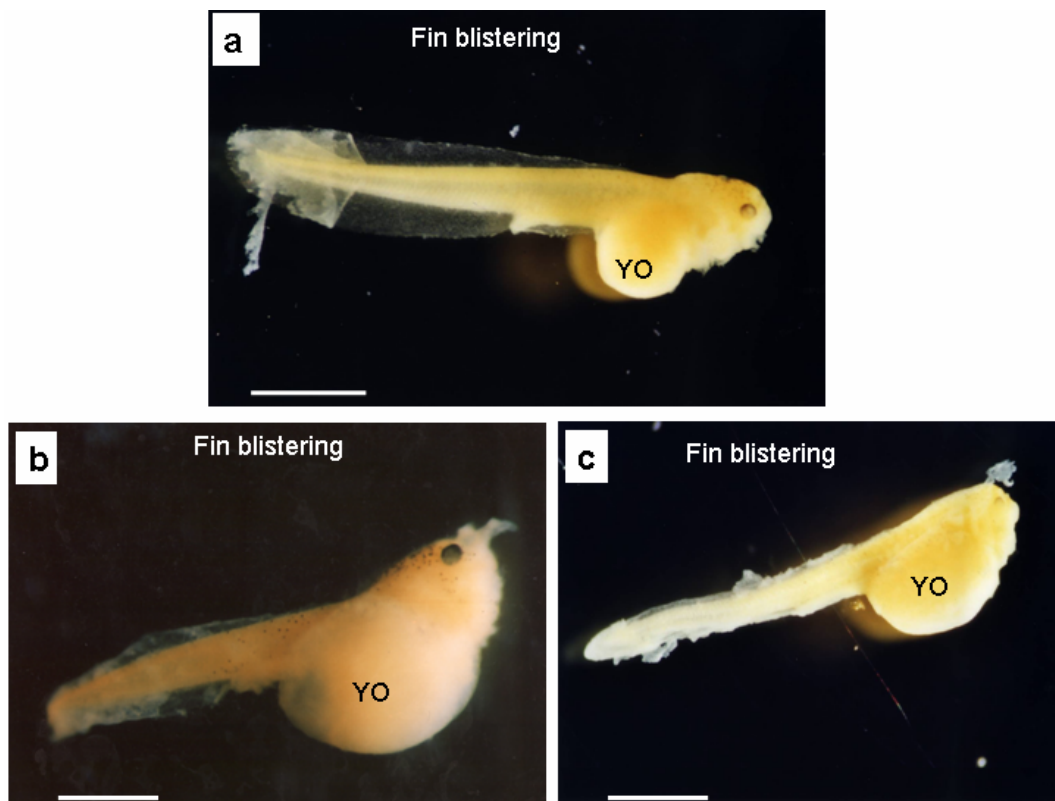


Fig. 26. Fin blistering /necrosis in the embryos of *Clarias gariepinus* after lead exposure (a) blistering of the posterior part of the tail (120h-PFS exposed to 100 $\mu\text{g/l}$ lead nitrate), (b) degeneration of the fin throughout the tail (120h-PFS exposed to 300 $\mu\text{g/l}$ lead nitrate), (c) blistering of the whole fin (120h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate). YO= yolk sac oedema, PFS=post fertilization stage. Scale bare= 1mm.

5.2.2.6 Reduced pigmentation

At 144h-PFS, when pigmentation covered the entire body proper in the control (Fig. 15f), a noticeable reduction in pigmentation of all treatment groups was observed (Fig. 26b, c). This was concentration dependent.

5.2.3 Histopathological changes in post-hatching stages

Four major histopathological categories comprising notochordal defect, gill malformations, eye malformations and detached skin were recorded. All these malformations were found only in the embryos exposed to 300 $\mu\text{g/l}$ and 500 $\mu\text{g/l}$ lead nitrate from 96h-PFS onward.

5.2.3.1 Malformations of the notochord

The notochord of embryos consisted of notochordal epithelium and the vacuolated cells, enclosed in a fibrous sheath (Fig. 27a, d). The main histological malformation recorded was an abnormal shaped notochord (Fig. 27b-c, e-f). The defected notochord was recorded in the embryos exposed to 300 $\mu\text{g/l}$ lead (Fig. 27b). A stronger malformations in the notochord were observed in the groups exposed to 500 $\mu\text{g/l}$ lead (Fig. 27c). This kind of malformation was observed in trunk (Fig. 27e) and tail regions (Fig. 27f).

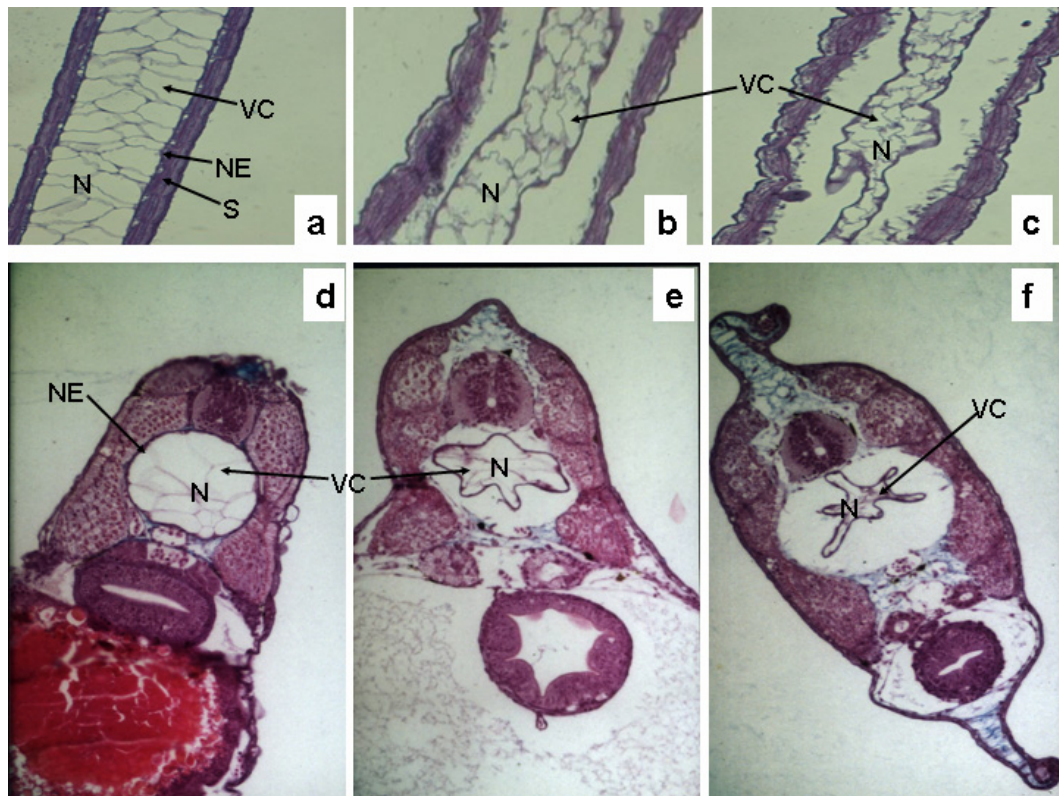


Fig. 27. Histological lesions of the notochord in the embryos of *Clarias gariepinus* after lead exposure (a) sagittal section through the notochord of 120h-PFS control (x100), (b) sagittal section through defected notochord of 120h-PFS exposed to 300 $\mu\text{g/l}$ lead nitrate (x100), (c) sagittal section through deformed notochord of 120h-PFS exposed to 500 $\mu\text{g/l}$ lead (x100), (d) transverse section through the trunk region of 120h-PFS control (x100), (e) transverse section through the trunk region of 120h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate (x100), (f) transverse section through the tail region of 120h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate (x100). N= notochord, VC=vacuolated cells, NE= notochordal epithelium, S= sheath, PFS=post fertilization stage. Staining: H&E and Alcian blue.

5.2.3.2 Malformations of the gills

In the control, interdigitating lamellae with well developed cartilagenous rods were observed. Fusion of adjacent lamellae was not recorded (Fig. 28a). At 168h-PFS the formation of the gill filaments started. In contrast, embryos exposed to the highest two lead dosages showed arrested development of the gill. The underdeveloped gills of the embryos exposed to 300 $\mu\text{g/l}$ lead had less skeletal muscle and reduced cartilage in the gill arch (Fig. 28b). A fusion of adjacent lamellae with failure to form gill filaments was recorded in the embryos exposed to 500 $\mu\text{g/l}$ lead (Fig. 28c, d).

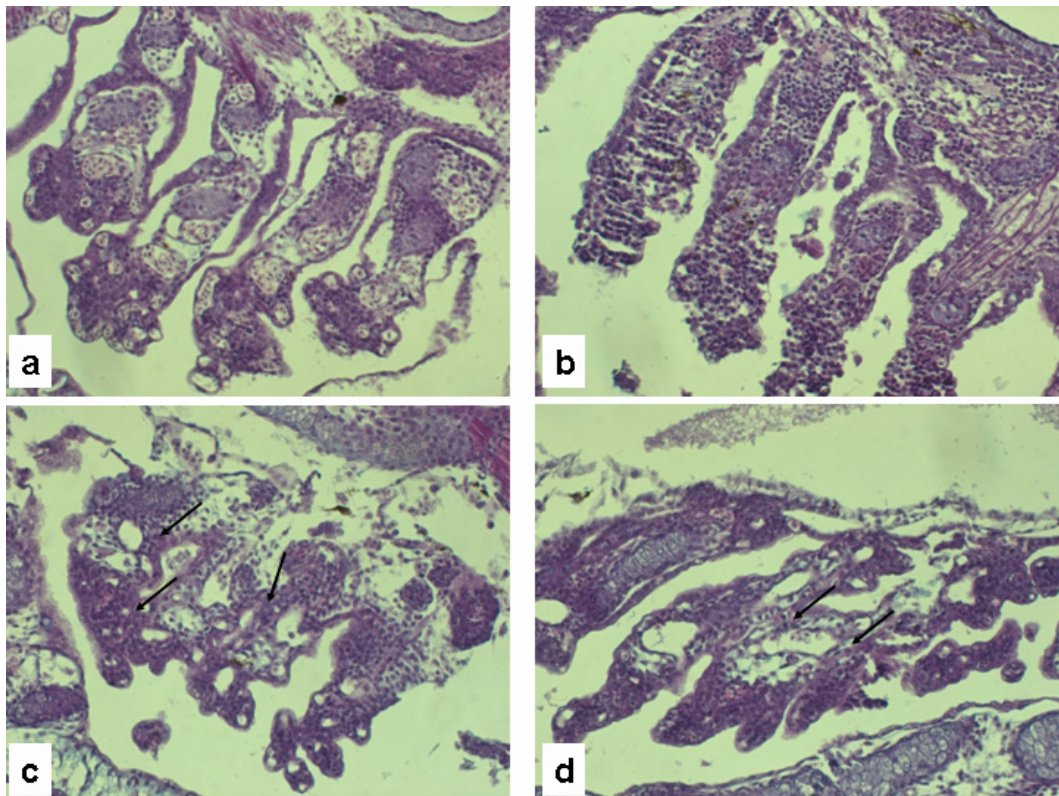


Fig. 28. Histological lesions of gills in the embryos of *Clarias gariepinus* after lead exposure (a) sagittal section through the gills of 144h-PFS control (x200), (b) sagittal section through the gills of 144h-PFS exposed to 300 $\mu\text{g/l}$ lead nitrate (x200), (c,d)) sagittal section of 144h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate showing fusion of adjacent lamellae (arrow) (x200). PFS=post fertilization stage. Staining: H&E and Alcian blue.

5.2.3.3 Malformations of the eyes

In the control, no malformations were observed and the retina consisted of uniform layers of retinal cells surrounded by a uniform pigmented epithelium (Fig. 29a). In contrast, the treatment groups exhibited different degrees of malformations including irregular and discontinuous pigmented epithelium, degeneration of retinal cells, and degeneration of the lens (Fig. 29b-f). At 96h-PFS, first abnormalities became obvious: In the groups exposed to 300 $\mu\text{g/l}$ and 500 $\mu\text{g/l}$ lead nitrate the pigmented cells were

non-uniform layered (Fig. 29b). In addition, in the groups exposed to 500 $\mu\text{g/l}$ lead, some retinal cells were lacking pigmentation and intact pigmentation was concentrated on the side opposite the lens (Fig. 29c). At the 120h-PFS degeneration in the outer nuclear layer and in the pigmented layer was recorded in the group exposed to 300 $\mu\text{g/l}$ and 500 $\mu\text{g/l}$ lead (Fig. 29d). The degeneration comprised all retinal layers at 144h-PFS exposed to 300 $\mu\text{g/l}$ and 500 $\mu\text{g/l}$ (Fig. 29e). In the group exposed to 500 $\mu\text{g/l}$ lead degeneration of the lens was additionally observed (Fig. 29f). Therefore, the degree of the effect increased with increasing lead concentration and with the length of the exposure time.

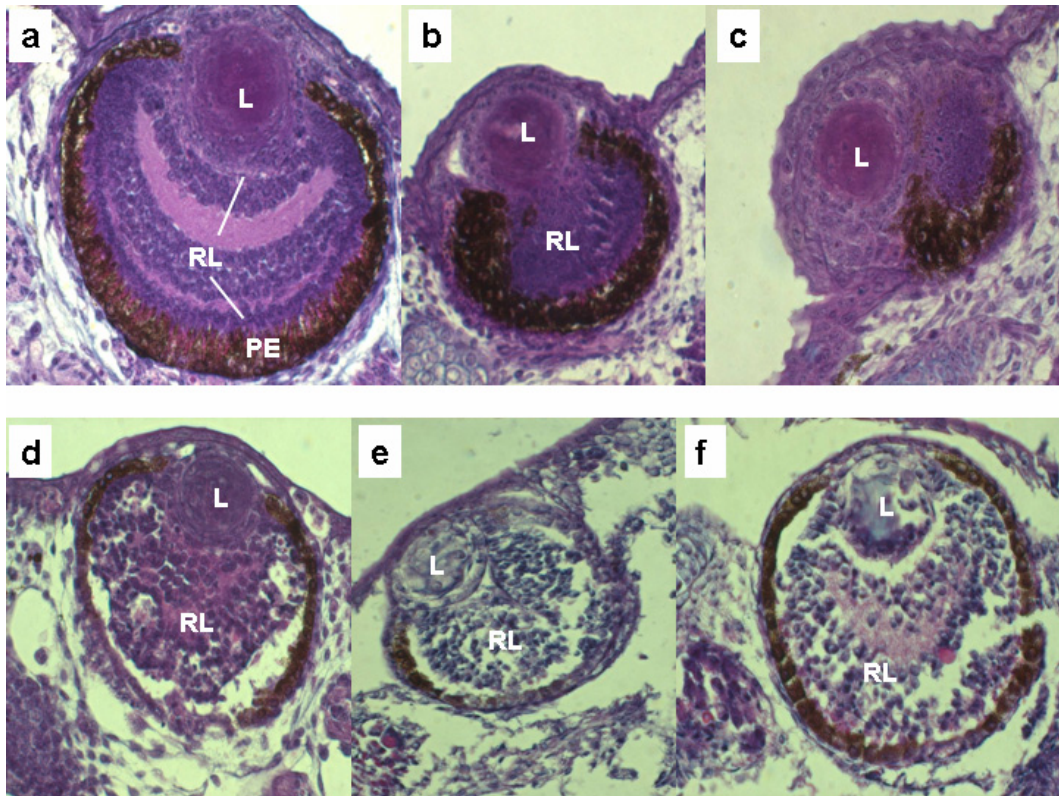


Fig. 29. Histological lesions of the eye in the embryos of *Clarias gariepinus* after lead exposure (a) sagittal section through the eye of 96h-PFS control (x400), (b) sagittal section through the eye of 96h-PFS exposed to 300 $\mu\text{g/l}$ lead nitrate (x400), (c) sagittal section through the eye of 96h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate (x400), (d) sagittal section through the eye of 120h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate (x400), (e) sagittal section through the eye of 144h-PFS exposed to 300 $\mu\text{g/l}$ lead nitrate (x400), (f) sagittal section through the eye of 144h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate (x400). RL= retinal layers, PE=pigmented epithelium, L=lens, PFS=post fertilization stage. Staining: H&E and Alcian blue.

5.2.3.4 Skin detached from the underlying muscles

In the control embryos, the skin was firmly attached to the well developed underlying muscles (Fig. 30a). After lead-exposure skin detached from the underlying muscles (Fig. 30b-c). This malformation was recorded only at the 120h- and 144h-PFS, it was most prominent in the highest lead concentration: In the group exposed to 300 $\mu\text{g/l}$ lead nitrate the skin detached only partially from its underlying muscles (Fig. 30b).

In 500 $\mu\text{g/l}$ treatment, skin was completely detached (Fig. 30c). The detachment of the skin was always associated with necrosis or degenerations in the underlying muscles. Again a dosage correlation was observed.

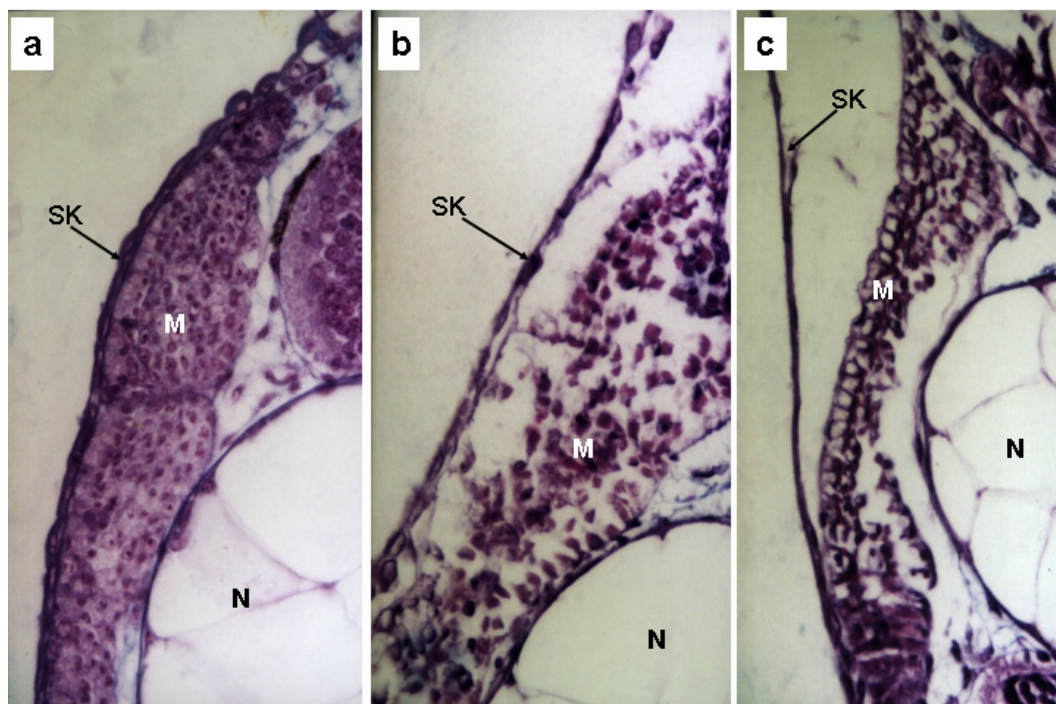


Fig. 30. Histological lesions of skin in the embryos of *Clarias gariepinus* after lead exposure. (a) Transverse section through the skin of 120h-PFS control (x400), (b) transverse section through the skin of 120h-PFS exposed to 300 $\mu\text{g/l}$ lead nitrate (x400), (c) transverse section through the skin of 120h-PFS exposed to 500 $\mu\text{g/l}$ lead nitrate (x400). SK=skin, M=muscle, N=notochord, PFS=post fertilization stage. Staining: H&E and Alcian blue.

5.2.4 Bioaccumulation of lead

Mean lead concentrations in whole embryos are shown in Table 4. The lowest concentration of lead was recorded in the control groups (0.3-1,0 $\mu\text{g/g}$ wet wt) which increased significantly ($p < 0,05$) with increasing lead dose in all stages (Table 4). Highest concentrations were found in the 30h-PFS (11.9 and 13.3 $\mu\text{g/g}$ wet wt)and 48h-PFS (12.2 and 16.1 $\mu\text{g/g}$ wet wt)after exposure to 300 and 500 $\mu\text{g/l}$ lead nitrate. A decrease of accumulated lead was observed with increasing exposure time. A significant correlation between accumulated lead and its concentration in the water was evident.

Table 4. Bioaccumulation of lead (means \pm SD) in different embryonic stages of *Clarias gariepinus* exposed to different concentrations of lead nitrate. N = 3

PFS= Post fertilization stage

* The hatching process started at 40 h post fertilization

Embryonic stages	Treatments ($\mu\text{g/l}$)	Lead concentration ($\mu\text{g/g wet wt}$)
30h-PFS (Prehatching stages)	Control	0,5 \pm 0,00
	100	3,2 \pm 0,11
	300	11,9 \pm 2,95
	500	13,3 \pm 0,03
48h-PFS*	Control	1,0 \pm 0,00
	100	4,1 \pm 0,19
	300	12,2 \pm 0,13
	500	16,1 \pm 0,09
96h-PFS	Control	0,5 \pm 0,11
	100	2,9 \pm 0,12
	300	3,7 \pm 0,16
	500	3,9 \pm 0,00
144h-PFS	Control	1,0 \pm 0,24
	100	1,6 \pm 0,25
	300	3,2 \pm 0,22
	500	6,6 \pm 0,22
168h-PFS	Control	0,3 \pm 0,00
	100	0,5 \pm 0,00
	300	1,1 \pm 0,00
	500	1,4 \pm 0,12

5.3 Discussion

In spite of numerous studies on heavy metal toxicity in fish, few investigate the effect on embryonic development (Devlin, 2006). Changes in the time scale of embryonic development are commonly observed. Depending on the metal and its concentration the development may be delayed or, less frequent, accelerated (Ernst and Neff, 1977; von Westernhagen, 1988). Nickel and copper accelerate the development of rainbow trout eggs by about 45 % and 20 %, respectively (McKim and Benoit, 1971; Shaw and Brown, 1971). Cadmium had a similar effects on herring embryos (von Westernhagen, 1974). However, reverse effect, prolonging incubation period caused by the same heavy metals, e.g. cadmium and copper (von Westernhagen, 1988). Nguyen and Janssen (2002) concluded that the exposure of the African catfish to some toxic substance (Cr, Cd, Cu, NaPCP and Malathion) caused non significant changes in the hatching time. In contrast, exposure of fertilized eggs to lead nitrate in the present work prolonged the incubation period and caused sever malformations. Delayed hatch may result from a retarded development or the inability of embryos to break the chorion (von Westernhagen, 1988). Physiologically, lead can also cause malfunctioning or even inhibition of egg shell digestive enzymes (chorionases) (Stouthart *et al.*, 1994). In the present work exposure to lead nitrate reduced the hatching success and the hatching rate from 75 % in the control group to 40 % in the group exposed to 500 µg /l lead. Thus, reduction in hatching rate is a sensitive toxicological parameter for monitoring sublethal lead exposure. In contrast, Niimi and Laham (1975) stated that hatching rate is not a reliable parameter because of the short life time of zebrafish embryo within the egg shell and reduced bioaccumulation.

Little information is available on lead-induced deformation in fish embryos and larvae (Jeziarska *et al.*, 2000). The early exposure to lead would have produced more sever defects (Weis and Weis, 1977). Such sever defects were recorded in our experiment in the form of irregular-shaped head and/or pericardial oedema of the newly hatched embryos exposed to 500 µg/l lead. Irregular head shape may even be specific for lead toxicity because it was observed for the fist time here. Pericardial oedema is a non specific as it is reported in response to other inorganic or organic pollutants (Hallare *et al.*, 2005). These two sever defects were lethal and led to the death of the malformed embryos few hours after hatching and were consequently not observed later on. A large or deformed yolk sac recorded here indicated metabolic or osmotic disturbances that

may be caused by mitochondrial malformation as suggested by von Westernhagen (1988). Yolk sac oedema were induced by several toxicant in a variety of fish larvae (Marty *et al.*, 1990; Walker and Peterson, 1991; Nguyen *et al.*, 1997; Jezierska *et al.*, 2000; Hallare *et al.*, 2005). Therefore, this type of malformation seems nonspecific (Nguyen *et al.*, 1997) and was, for example, recorded in the embryos of *C. gariepinus* after malathion exposure (Nguyen and Janssen, 2002) and in zebrafish embryo after cadmium exposure (Hallare *et al.*, 2005). The lead exposure induced metabolic and osmotic disturbances subsequently prevent the proper use of the energy stored in the yolk as suggested by the delayed hatch. Such disturbances often result in the hydration of the front portion of the yolk sac (Linden, 1978; Alderdice *et al.*, 1979) and lead subsequently to yolk sac oedema. The yolk sac is the site of early blood flow and extensive vascular remodeling during early development (Kimmel *et al.*, 1995; Henry *et al.*, 1997). However, lead did not affect early morphogenesis and yolk sac oedema was not observed until 120h-PFS.

Different kinds of notochordal curvature were recorded during our experiments including lordosis, scoliosis, kyphosis and C-shaped body. Damage of the vertebral column expressed as curvature of the larval body axis is caused by all heavy metals toxification (Ozoh, 1979; Stouthart *et al.*, 1994; Cheng *et al.*, 2000; Jezierska *et al.*, 2000; Nguyen and Janssen, 2002; Hallare *et al.*, 2005). Lordosis, scoliosis, kyphosis and C-shaped body have been previously reported after lead exposure in different fish species comprising zebrafish (Ozoh, 1979) brook trout (Holcombe *et al.*, 1976) and common carp (Stouthart *et al.*, 1994; Jezierska *et al.*, 2000). Skeletal defects in lead-treated embryos are also common abnormalities caused by a variety of teratogens (Battle and Hisaoka, 1952; Anderson and Battle, 1967; Weis and Weis, 1977). In conclusion, such malformations are nonspecific for a metal toxicity. Since no elements were recorded in the unconstricted notochord, the irregular spasms of the body musculature may act directly on the notochord, resulting in a bending of the body observed here (Nguyen *et al.*, 1997). In concordance, Cheng *et al.* (2000) attributed the notochordal deformities to a reduction in myosin and myotom development. Consequently, spatial alterations of the notochord affect ontogeny twice: Firstly, the neural tube, covering the dorsal face of the notochord, will be deformed as well. Secondly, the deformed notochord most probably will interfere with the normal development of the vertebrae, as the development of the bony vertebral centra occur

around the notochordal sheath (Nguyen *et al.*, 1997). Since the frequencies of the gross malformations decreased in time, we conclude a lethal impact and selected mortality of abnormal embryos in a agreement with von Westernhagen (1988) and Klumpp and von Westernhagen (1995).

An array of minor deformities can be caused by pollutants (von Westernhagen, 1988). The most common minor deformities are the finfold defect (blistering or necrosis) and the reduction in pigmentation. Fin erosion and fin defect have been previously reported in different fish species (von Westernhagen *et al.*, 1975; Ozoh, 1979; Cheng *et al.*, 2000; Hallare *et al.*, 2005). Cheng *et al.* (2000) postulated that tail erosion can be explained genetically as resulting from the inability of treated embryos to expresses the *evenskipid* gene, which is important during tail development. Fin blistering or fin necrosis recorded in the present work were similar to that observed by Ozoh (1979) and Hallare *et al.* (2005) in zebrafish embryos. The finfold defect reported in our experiment suggests a sublethal impact. A reduction in pigmentation was reported in *C. gariepinus* by Nguyen and Janssen (2002) and in *Danio rerio* by Ozoh (1980) due to the exposure to lead. Pigmentation changes in fish are often stress-induced (Ozoh, 1980; Nguyen and Janssen, 2002). Pigmentation of the skin is controlled by Melanocyte Stimulating Hormone (α MSH) and Melanin-Concentrating Hormone (MCH), which are known to be up regulated during stress response. Reduction in pigmentation of *C. gariepinus* may either result from stress at the cellular, organ or individual level or alter the function of α MSH and MCH directly.

Embryo pathology, including histopathology, provides a reliable, easily quantifiable index of low-level toxic stress to a broad range of environmental pollutants (Devlin, 2006). Unlike the morphological malformation, histopathological changes were only recorded in embryos exposed to highest dosages (300 μ g/l, 500 μ g/l lead nitrate). Thus, the morphological malformations were more sensitive for monitoring lead toxicity than the histopathological abnormalities. In fish, direct transport across the gills seems to be primary mode of uptake (Merlini and Pozzi, 1977; Hodson *et al.*, 1978). Therefore, gills are intensely exposed as indicated by reduced development of cartilage and lamellae and the fusion of adjacent lamellae. Theses changes impair respiratory function due to the surface reduction available for gas exchange (Conklin *et al.*, 1992). Malformations of the notochord – even if sublethal- impair functionality and reduce

swimming ability as observed here. Furthermore, skin detachment may increase rheostatic resistance. Like most histological lesions skin detachment and its associated muscle necrosis is non-specific to lead and was observed in response to other pollutant (Ernst and Neff, 1977; Olaifa *et al.*, 2003).

In most fishes species vision is considered the dominant sense during early life stages because it is essential for feeding, orientation, schooling and avoiding potential predators (Rodriguez and Gisber, 2001; Garcia-Abiado *et al.*, 2006). Therefore, sublethal abnormalities may easily result in mortality at a later stage, particularly in the natural environment. Normally developed eyes of *C. gariepinus* were observed two days after hatching consisting of a pigmented epithelium, two nuclear layers, two plexiform layers, a ganglion layer and a lens. Unlike mammalian eyes, finfish eyes do not have eyelids so they are constantly exposed to the water and toxicant present in the water have direct or indirect access to the eye (Garcia-Abiado *et al.*, 2006). Since internal tissues are not exposed directly to the external environment only few distinct organs are highly affected.

The present results show that lead toxicity was dosage dependent in African catfish embryos, this is congruent with other results, for example in gold fish *Carassius auratus* (Zhang *et al.*, 2005). Lead accumulated mainly in the chorion (Stouthart *et al.*, 1994) as suggested here by the highest concentration in the 30h-PFS and 48h-PFS stages. Accumulation of lead in the chorion suggests that, the chorion acts as an effective barrier protecting the embryo.

CHAPTER 6

Effects of Lead nitrate on the activity of the metabolic enzymes G6PDH, LDH and PK

6.1 Introduction

Biochemical and physiological indicators such as enzymes, could be used (as biomarkers) to identify possible environmental contaminations before the health of aquatic organisms is seriously affected (Jiminez and Stegeman, 1990; Barnhoorn and van Vuren, 2004) and to develop water quality indices (Powers, 1989; Gayet *et al.*, 1993; Zollner, 1993; Melancon, 1995; Pickering and Pottinger, 1995). Such a biochemical approach has been advocated to provide an early warning of potentially damaging changes in stressed fish (Casillas *et al.*, 1983). In toxicological studies of acute exposure, changes in concentrations and activities of some enzymes may reflect cell damage in specific organs (Casillas *et al.*, 1983; Heath, 1996).

The enzymes Glucose-6-Phosphate Dehydrogenase (G6PDH), Lactate Dehydrogenase (LDH) and Pyruvate Kinase (PK) are metabolic key factors (Ocampos *et al.*, 1987; Gill *et al.*, 1990; Coppes, 1992; Pelletier *et al.*, 1994; Gilli *et al.*, 2000; Kurutas and Tuncer, 2000; Leopold and Loscalzo, 2000; Long *et al.*, 2003). It has been traditionally thought that G6PDH was a typical 'housekeeping' enzyme that was regulated solely by the ratio of NADPH to NAD (Kletzien *et al.*, 1994; Tian *et al.*, 1999). G6PDH has long been recognized as an antioxidant enzyme (Nogae and Johnston, 1990; Pandolfi *et al.*, 1995; Salvemini *et al.*, 1999) and as a biomarker of pollution-induced carcinogenesis in fish (Winzer *et al.*, 2001). It has been postulated that heavy metals have different effects on the activity of G6PDH in different fish species (Strik *et al.*, 1975; Barnhoorn, 1996).

The cytoplasmic enzyme LDH is widely used as marker of organ or tissue lesions in toxicology and in clinical chemistry (Das *et al.*, 2004a). It has been used for demonstrating tissue damage in fish (Das *et al.*, 2004a). In most cases of tissue damage, whether due to a disease or a toxic compound, the activity of LDH was reported to be significantly affected (Singh and Sharma, 1998). LDH is a source of the oxidised coenzyme during the period of transient anaerobiosis or a reduced form of such

coenzyme during aerobiosis (Coppes, 1992). Therefore, LDH has been also used as indicative of hypoxic conditions in the organism (Das *et al.*, 2004a). Different metals seem to influence the activity of LDH (Christensen *et al.*, 1982; Gill *et al.*, 1990). G6PDH with LDH play an important role during glycolysis and they have direct effects on the development of fish (Shaklee *et al.*, 1974).

PK controls and catalysis the conversion of phosphoenol pyruvate (PEP) to pyruvate while regenerating ATP (Gilli *et al.*, 2000). Three forms of PK have been isolated in fish, and they show a variation in activity and occurrence between species. Pyruvate Kinase is an enzyme that is situated in the cytosol compartments in the cell and, therefore, is not as protected as the enzymes in the mitochondria. Several authors (Randall and Anderson, 1975; Ocampos *et al.*, 1987; Isani *et al.*, 1994) studied the inhibitory effect of divalent ions (Cu, Co, Be, Zn) on the activity of PK in fishes. Metals such as copper, manganese and iron have been found to alter the activity of PK (Hansen *et al.*, 1992; Barnhoorn, 1996).

The altered activities of G6PDH, LDH and PK could be useful biomarkers of water pollution. If the organism is exposed to pollutants, these enzymes respond by activation or deactivation. The effect of heavy metals on enzyme activities have been described in fishes (Jiminez and Stegeman, 1990). Most of these studies addressed the impact of heavy metal exposure on the enzyme activities of juveniles (Almeida *et al.*, 2001; Almeida *et al.*, 2002; Das *et al.*, 2004a) and adult fishes (Jackim *et al.*, 1970; Sastry and Gupta, 1980; Gupta and Sastry, 1981; Christensen *et al.*, 1982; Gill *et al.*, 1990; Bainy *et al.*, 1996; Almeida *et al.*, 2001; Almeida *et al.*, 2002; Barnhoorn and van Vuren, 2004). However, only two articles (Christensen, 1975; Das *et al.*, 2004a) described the effect of chemicals on the enzyme activities of fish embryos. Although several researchers studied the effect of water pollution on the physiology and biochemistry of fish, no detailed information is available regarding the effects of lead on the activity of G6PDH, LDH and PK during early developmental stages of fish. So, the present work aims to study the ontogenetic variations in the activities of the metabolic enzymes (G6PDH, LDH and PK) during early development (endogenous feeding) of *Clarias gariepinus* and to use these early developmental stages as model system to evaluate the effect of lead nitrate on the activity of these enzymes.

6.2 Results

In order to study the effect of different concentrations of lead nitrate on the activities of G6PDH, LDH and PK, we first had to study the ontogenetic changes in the activities of these enzymes during normogenesis.

6.2.1 The normal ontogenetic variations in enzyme activities:

The activities of G6PDH, LDH and PK showed variability during the early embryonic stages (30h-168h PFS) under normal conditions (Fig. 31). The pattern of the ontogenetic variation exhibited a significant ($p < 0.05$) decrease in the activities of all three enzymes till 96h-PFS. After 96h-PFS the activities of G6PDH and LDH increased toward the higher stages (144h-168h PFS). This increase was insignificant in the case of G6PDH and significant ($p < 0.05$) in the case of LDH (Fig. 31). PK activity (after 96h-PFS) showed significant ($p < 0.05$) increase till 144h-PFS and then a significant ($p < 0.05$) decrease till 168h-PFS (Fig. 31). Therefore, the activities of all three enzymes decreased from 30h-PFS to 96h-PFS, followed by an increase in the activities of G6PDH and LDH. PK showed significant fluctuations in its activity (Fig. 31).

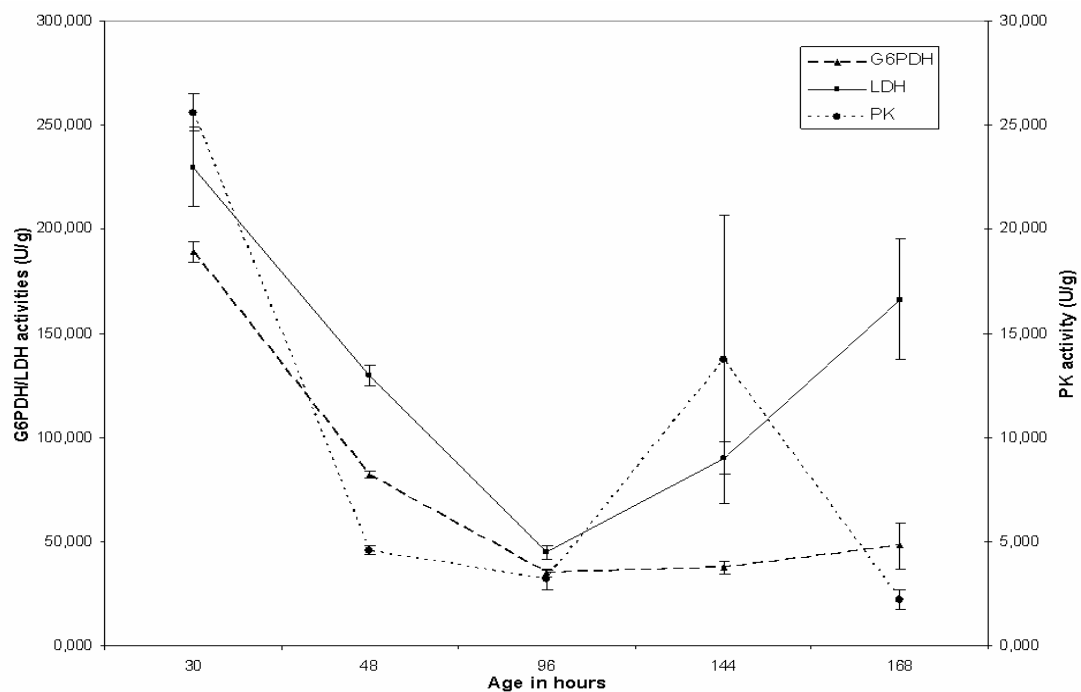


Fig. 31. Pattern of the activities of Glucose-6-Phosphate Dehydrogenase (G6PDH), Lactate Dehydrogenase (LDH) and Pyruvate Kinase (PK) (mean \pm SE) of the early developmental stages (during the period of endogenous feeding) of *Clarias gariepinus* under controlled condition.

6.2.2 The enzyme activities after exposure to lead nitrate in comparison with normogenesis:

The patterns of the enzyme activities of the different early ontogenetic stages under the influence of three different lead nitrate doses are presented in Tables 5, 6 and 7. In the groups exposed to 100 µg/l lead nitrate the activities of G6PDH was significantly lower ($p < 0.05$) till 96h-PFS, then the activity was insignificantly higher till 168 h-PFS stage (Tables 5). In the groups exposed to 300 µg/l lead nitrate the activity of G6PDH decreased significantly ($p < 0.05$) till 96h-PFS and increased significantly till 168h-PFS (Tables 5). The activity of such enzyme in the groups exposed to 500 µg/l lead nitrate decreased insignificantly till 96h-PSF and then incread significantly ($p < 0.05$) till 168h-PFS (Tables 5). In the case of LDH the activity was significantly lower ($p < 0.05$) till 96h-PFS, then the activity was significantly ($p < 0.05$) higher till the 168h-PFS stage (Tables 6) in the groups exposed to 100 µg/l lead nitrate. In the groups exposed to 300 µg/l and 500 µg/l lead nitrate the activity of LDH decreased significantly ($p < 0.05$) till 96h-PFS (Tables 6). Thereafter, the activity showed significant increase ($p < 0.05$) till 168h-PFS (Tables 6). The activity of PK decreased significantly ($p < 0.05$) from 30h-PFS till 168h-PFS in the groups exposed to 100 µg/l lead nitrate. In the groups exposed to the other two concentrations (300 µg/l and 500 µg/l lead nitrate) the PK activity exhibited significant ($p < 0.05$) fluctuations towards increase and decrease (Table 7). In conclusion, the patterns of activity of the lead nitrate-treated embryos exhibited a pattern of enzyme activity similar to that of the untreated embryos (especially for G6PDH and LDH) but with significant variability in magnitude (Tables 5-7).

Different patterns of enzyme activities were recorded within each embryonic stage due to the exposure to different concentrations of lead nitrate in comparison with the control. In 30h-PFS (pre-hatching stage) the activities of the three selected enzymes exhibited similar patterns (Table 5, 6 and 7). The activities increased significantly ($p < 0.05$) with 100 µg/l lead nitrate dose recording the highest value of activity during the whole experiment (227.2, 295 and 27.6 unit/g protein for G6PDH, LDH and PK, respectively) and then the activities of the three enzymes decreased concomitantly with the higher doses (Tables 5, 6 and 7).

Table 5. Effect of different concentrations of lead nitrate on the activity of Glucose-6-Phosphate Dehydrogenase (G6PDH) [mean \pm SE] during early developmental stages of the African catfish *Clarias gariepinus*.

Embryonic stages*	Control	100 μ g/l	300 μ g/l	500 μ g/l
30h-PFS (prehatching)	189,2 \pm 5,0 (158,1-195,0) a (A)	227,2 \pm 6,8 (221,5-234,7) a (B)	149,6 \pm 3,2 (145,4-152,0) a (C)	133,0 \pm 14,4 (118,9-145,4) ab (C)
48h-PFS**	81,8 \pm 1,7 (79,3-82,6) b (A)	95,9 \pm 11,5 (85,9-105,7) b (B)	104,1 \pm 1,9 (102,4-105,7) b (BC)	109,9 \pm 5,6 (102,4-115,7) a (C)
96h-PFS	34,7 \pm 1,9 (33,0-36,4) c (A)	55,4 \pm 1,6 (52,8-56,2) c (AB)	67,8 \pm 14,4 (59,5-89,2) c (B)	106,6 \pm 23,3 (76,0-132,2) a (C)
144h-PFS	37,2 \pm 3,2 (33,0-39,7) cd (A)	52,9 \pm 4,7 (46,2-56,2) c (AB)	69,4 \pm 2,7 (66,1-72,7) c (B)	147,9 \pm 18,2 (132,2-165,3) b (C)
168h-PFS	47,9 \pm 11,0 (33,0-56,2) d (A)	61,1 \pm 9,5 (52,9-69,4) c (A)	104,1 \pm 7,9 (95,9-112,4) b (B)	162,8 \pm 1,7 (161,9-165,3) b (C)

PFS = Post fertilization stage.

*Embryonic stages of similar small letters are insignificant within the treatment at 0.05 levels (vertical comparison). Treatments of similar capital letters are insignificant within the embryonic stages at 0.05 levels (horizontal comparison).

** The hatching process started at 40 hour post fertilization.

Table 6. Effect of different concentrations of lead nitrate on the activity of Lactate Dehydrogenase (LDH) [mean \pm SE] during early developmental stages of the African catfish *Clarias gariepinus*.

Embryonic stages*	Control	100 μ g/l	300 μ g/l	500 μ g/l
30h-PFS	229,7 \pm 19,2	295,0 \pm 40,7	242,1 \pm 7,3	244,6 \pm 14,0
(prehatching)	(211,5-247,9) a (A)	(234,7-323,9) a (B)	(234,7-251,2) a (A)	(228,1-257,8) a (A)
48h-PFS**	129,7 \pm 4,9	114,9 \pm 14,6	95,8 \pm 2,7	90,1 \pm 11,9
	(125,6-135,5) b (A)	(99,2-129,9) b (AB)	(92,5-99,2) b (BC)	(72,7-99,2) b (C)
96h-PFS	44,6 \pm 3,3	41,3 \pm 1,9	36,4 \pm 2,7	35,5 \pm 3,1
	(42,9-49,6) c (A)	(39,7-42,9) c (AB)	(33,1-39,7) c (B)	(33,1-39,1) c (B)
144h-PFS	90,1 \pm 7,8	65,3 \pm 3,2	62,0 \pm 3,2	47,1 \pm 12,8
	(79,3-95,8) d (A)	(62,8-69,4) c (B)	(59,5-66,1) d (BC)	(33,1-59,5) c (C)
168h-PFS	166,1 \pm 28,9	122,3 \pm 9,7	109,1 \pm 14,0	81,8 \pm 12,5
	(132,2-191,7) e (A)	(112,2-135,5) b (B)	(95,9-128,9) b (BC)	(72,7-99,2) b (C)

PFS = Post fertilization stage.

*Embryonic stages of similar small letters are insignificant within the treatment at 0.05 levels (vertical comparison). Treatments of similar capital letters are insignificant within the embryonic stages at 0.05 levels (horizontal comparison).

** The hatching process started at 40 hour post fertilization.

Table 7. Effect of different concentrations of lead nitrate on the activity of Pyruvate Kinase (PK) [mean \pm SE] during early developmental stages of the African catfish *Clarias gariepinus*.

Embryonic stages*	Control	100 μ g/l	300 μ g/l	500 μ g/l
30h-PFS (prehatching)	25,6 \pm 0,9 (25,0-26,2) a (A)	27,6 \pm 2,0 (26,2-29,1) a (A)	7,6 \pm 1,3 (6,6-8,5) a (B)	3,9 \pm 0,7 (3,5-4,4) ac (B)
48h-PFS**	4,6 \pm 0,2 (4,4-4,7) b (A)	8,5 \pm 0,4 (8,2-8,8) b (B)	4,3 \pm 0,2 (4,1-4,4) bc (A)	7,4 \pm 0,2 (7,3-7,6) b (B)
96h-PFS	3,2 \pm 0,4 (2,8-3,5) b (A)	8,5 \pm 0,4 (8,2-8,8) b (B)	7,2 \pm 0,4 (7,0-7,6) a (B)	5,4 \pm 0,4 (5,1-5,7) c (C)
144h-PFS	13,7 \pm 6,9 (8,8-18,6) ab (A)	3,0 \pm 0,2 (2,8-3,2) c (A)	2,2 \pm 0,4 (1,9-2,5) c (A)	2,8 \pm 0,4 (2,5-3,2) a (A)
168h-PFS	2,2 \pm 0,4 (1,9-2,5) b (A)	2,8 \pm 0,0 (2,8-2,8) c (A)	5,5 \pm 0,7 (5,1-6,0) ab (B)	4,7 \pm 0,4 (4,4-5,1) c (B)

PFS = Post fertilization stage.

*Embryonic stages of similar small letters are insignificant within the treatment at 0.05 levels (vertical comparison). Treatments of similar capital letters are insignificant within the embryonic stages at 0.05 levels (horizontal comparison).

** The hatching process started at 40 hour post fertilization.

In the subsequent post-hatching stages (48h–168h-PFS) the activity of G6PDH increased significantly ($p < 0.05$) from the control up to 500 $\mu\text{g/l}$ lead exposure (Table 5). The activity of LDH was significantly inhibited ($p < 0.05$) with increasing concentration of lead nitrate during all post-hatched stages (48h–168 PFS) from the control to 500 $\mu\text{g/l}$ lead (Table 6). The PK activity in each of the post-hatching stages (48h–168h-PFS) showed significant and some times insignificant fluctuations (increase and decrease) with increasing lead doses (Table 7). The same patterns of differences between the control and the lead nitrate-treated groups were also obtained when the Dunnett.- T test was used (Table 8). In conclusion, in the 30h-PFS, the activities of G6PDH, LDH and PK increased due to exposure to 100 $\mu\text{g/l}$ lead and then decreased with increasing lead doses. After 30h-PFS, the patterns of the activity under stress varied, G6PDH increased, LDH decreased and PK showed fluctuations.

Table 8. Comparison between the activities of Glucose-6-Phosphate Dehydrogenase (G6PDH), Lactate Dehydrogenase (LDH) and Pyruvate Kinase (PK) in the control and in the other lead nitrate-treated groups during early developmental stages of the African catfish *Clarias gariepinus* using Dunnett-T test.

Enzymes	30h-PFS			48h-PFS			96h-PFS			144h-PFS			168h-PFS		
	100μ/l	300μ/l	500μ/l	100μ/l	300μ/l	500μ/l	100μ/l	300μ/l	500μ/l	100μ/l	300μ/l	500μ/l	100μ/l	300μ/l	500μ/l
G6PDH	*	*	*	*	*	*	--	*	*	--	*	*	--	*	*
LDH	*	--	--	--	*	*	--	*	*	*	*	*	*	*	*
PK	--	*	*	*	--	*	*	*	*	--	--	--	--	*	*

(*) Significant difference with the control (--) no difference with the control.

6.3 Discussion

6.3.1 The normal ontogenetic variations in enzyme activities:

There is no literature available concerning the pattern of ontogenetic metabolic enzyme activities in early developmental stages of fish before exogenous feeding. Only ontogenetic variations during development after the start of exogenous feeding were studied (Somero and Childress, 1985; Clarke *et al.*, 1992; Segner and Verreth, 1995). In the present work, the enzymatic activities of G6PDH, LDH and PK exhibited variability during the early embryonic stages of *C. gariepinus* (30-168h-PFS), a period that included pre-hatching and post-hatching intervals. The patterns of variations exhibited significant decreases in the enzyme activities of all three enzymes studies till 96h-PFS, followed by a relative overall significant increase in G6PDH and LDH activity ; the activity of the latter enzyme increased faster than that of G6PDH. PK activity after 96h-PFS showed significant fluctuation. The decrease of activities till 96h-PFS concerning all three enzymes led us to the conclusion that the embryo in its early embryonic stages depended on the maternal enzymes. After this stage, the zygotic genes began to work, as expressed by the increasing activities of these enzymes (Mekkawy and Lashein, 2003). A similar utilisation of the maternal G6PDH and LDH enzyme stores and their subsequent degradation before zygotic translation of mRNA was postulated by Mekkawy and Lashein (2003) for the grass carp, *Ctenopharyngodon idellus*. Those authors, on a genetic basis, concluded that the zygotic genes of the *C. idellus* embryo appear to be inactive up to the process of organogenesis. In *C. gariepinus* there is still such inactivation up to 96h-PFS, during which time the major switching process between maternal and zygotic gene activation occurs. This means the utilisation of maternal mRNA enzymes for a long period in comparison with *C. idellus* (Mekkawy and Lashein, 2003).

Increase of the G6PDH and LDH activities from 96h-PFS (2 days after hatching) onward, before the onset of exogenous feeding (at 196h-PFS of *C. gariepinus*) referred to the early substantial capacity for NADPH generation based on switch on zygotic gene mechanism (Segner and Verreth, 1995). Segner and Verreth (1995) referred to such a capacity existing from the onset of exogenous feeding onward, as evidenced by the increase of the G6PDH activity with age. Many authors referred to the increase of LDH and G6PDH enzyme activities with increasing larval age of different fish species

(Pelletier *et al.*, 1995; Nathanailides, 1996). However, Nathanailides (1996) referred to the decrease of LDH activity during development of *Tilapia* larvae. The PK-activity as index of anaerobic potential (Forstner *et al.*, 1983; Hinterleitner *et al.*, 1987; Kiessling *et al.*, 1991) showed significant fluctuations at the onset of zygotic gene mechanism (96h-PFS).

6.3.2 The enzyme activities after exposure to lead nitrate:

The ontogenetic pattern of the activity of the lead nitrate-treated embryos exhibited a pattern of enzyme activities similar to that of the normal embryos but with a significant variability in magnitude. However, within each embryonic stage, in comparison with the control, different patterns of enzyme activities were recorded. At 30h-PFS, G6PDH activity increased at 100 µg/l lead exposure; with higher doses the activity decreased significantly in comparison with the control. In each of the other developmental stages (48h – 168h- PFS) the G6PDH activity increased significantly compared to the control up to 500 µg/l lead exposure. A similar increase in G6PDH activity under stress were previously recorded (Wu and Lam, 1997; Stephensen *et al.*, 2000; Pandey *et al.*, 2003; Rosety-Rodriguez *et al.*, 2005). Pandey *et al.* (2003) recorded an increase in G6PDH activity in muscles, liver and gills of the Indian freshwater fish *Wallago attu* with increasing pollution in the river Yamuna. Stephensen *et al.* (2000) demonstrated that fishes from polluted sites have high G6PDH activity due to the increased production of NADH for the detoxification process. Rosety-Rodriguez *et al.* (2005) also recorded that the activity of G6PDH increased in the blood of gilthead fish at the two high concentrations of malathion tested. Glutathione (GSH) serves to protect the cell against oxidative damage as it conjugates with compounds of exogenous and endogenous origin (Singhal *et al.*, 1987). Thus, a decrease in the cellular level of GSH may cause accumulation of reactive oxygen species (ROS). The increase in G6PDH activity represents protection against elevated levels of ROS in cells exposed to an oxidant stress through the increased production of NADH (Stephensen *et al.*, 2000; Leopold *et al.*, 2003).

LDH is generally associated with the cellular metabolic activity. Such activity is inhibited under stress especially after exposure to heavy metals (Singh *et al.*, 1974; Sastry and Gupta, 1980; Gupta and Sastry, 1981; Sastry and Rao, 1984; Mishra and Shukla, 1997; Fennouh *et al.*, 1998; Singh and Sharma, 1998; Almeida *et al.*, 2001;

Elumalai *et al.*, 2002). In *C. gariepinus* at 30h-PFS, the 100 µg/l lead nitrate dose lead to a significant increase in the LDH enzyme activity. No significant effects with higher doses of lead have been recorded in comparison with the control during this stage. In other embryonic stages (48h – 168h-PFS) the enzyme activity significantly decreased concomitantly with the lead dose. In other words, the activity of LDH was significantly inhibited with increasing concentrations of lead nitrate. Sastry and Gupta (1980) postulated that inhibition in activity may be due to (i) ion imbalance or to (ii) intracellular action of metal subsequent to initial plasma membrane damage. This inhibitory effect may also be due to the direct binding of the lead to the enzyme (Rajanna *et al.*, 1990) and the formation of an enzyme-inhibitor complex (Singh and Sharma, 1998). In consistence with our results Mishra and Shukla (1997) reported that endosulfan (an insecticide) inhibited the activity of LDH in muscles and liver of the catfish *Clarias batrachus*. Elumalai *et al.* (2002) recorded inhibition in LDH activity in the crab *carcinus maenas* due to exposure to Cu, Cr and a mixture of both. In the same way Sastry and Rao (1984) and Almeida *et al.* (2001) recorded inhibition of the activity of LDH in the freshwater Murrel *Channa punctatus* due to exposure to mercuric chloride and in Nile tilapia *Oreochromis niloticus* due to the exposure to cadmium. In contrast to the present results, Das *et al.* (2004a) recorded an increase in the activity of LDH in the liver, gill, kidney, brain and serum of fingerlings of three species of the Indian major carps (*Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*) due to exposure to nitrite toxicity.

Most of the egg's carbohydrates are associated with the egg membrane and, therefore, are probably unavailable to the developing embryo, at least until hatching, the time of their release from that membrane (Mekkawy and Lashein, 2003). However, intensive catabolism of carbohydrates commences at fertilization indicating that carbohydrates play an important nutritive role during initial cleavage (Diwan and Dhakad, 1995). Such very early metabolism of carbohydrates is reflected in the present work in terms of the decreased activity of LDH and the increased activity of the G6PDH enzyme system. It is supposed that the synthesis of metabolic enzymes through activation of maternal genes and internal carbohydrate metabolism is an adaptive or antioxidant mechanism of the embryo to tolerate the lead effects.

PK is a cytosolic enzyme that can be taken as indicator of the capacity of muscle tissue for anaerobic function (Berges and Ballantyne, 1991; Lemos *et al.*, 2003). In the

present work the PK activity in 30h-PFS insignificantly increased at lead dose of 100 µg/l, and then sharply decreased. In each of the other embryonic stages (48h – 168h-PFS), PK activity showed significant fluctuations with the increasing lead doses. No data are available to explain such a specific trend of the activity of PK due to the exposure to different concentrations of lead nitrate.

Does the interaction between the two factors (age and lead nitrate dose) maximize the impacts on the enzyme activities? The interaction and the main effects of the age and lead-dose were found to be highly significant referring to their great impact of lead on these enzyme systems with increasing age.

No specific response in the activities of all three enzymes was recorded in the pre-hatching stage (30h-PFS) after exposure to the lead nitrate doses. This means the lead nitrate had a minute effect on the activities of these enzymes at this stage. In conclusion, the low susceptibility in the pre-hatching stages is most probably a consequence of the chorion, which seems to protect the embryo from a wide range of pollutants. Congruently, Osman et al. (unpublished) recorded that lead has a negligible effect on the degree of DNA damage in the pre-hatching stage (30h-PFS) in comparison with the post-hatching stages (48h-168h-PFS) due to the presence of the chorion, which protect the embryos. Mekkawy and Osman (2006) postulated that the chorion of *C. gariepinus* started the hardening process 3 h after fertilization providing protection from lead 6 h after fertilization. This hypothesis was first suggested by Nguyen *et al.* (1999) with regard to heavy metal accumulation but is now further supported by the findings on reduced genotoxicity in pre-hatching stages. The same result has been recorded by Honkanen (2004) suggesting that the presence of an egg shell in pre-hatching stages seems to protect the embryos from a wide range of external stress factors during critical developmental events.

It could be demonstrated that the activities of G6PDH and LDH gave specific patterns of responses (G6PDH increased and LDH decrease) due to the exposure to different lead nitrate doses during the post hatching stages (48h – 168h-PFS). PK enzyme did not give a specific trend of response (increase or decrease) due to the exposure to the lead nitrate doses. In conclusion, the measurements of G6PDH and LDH but not PK activities could be useful biomarkers of lead pollution to reveal

embryotoxic potential in fish; the post hatching stages of the African catfish *C. gariepinus* are the most sensitive in early development.

CHAPTER 7

Monitoring of DNA breakage after exposure to lead nitrate

7.1 Introduction

During the last years, several techniques to detect directly DNA damage - as opposed to the biological effects (micronuclei, structural chromosomal aberrations, etc.) that result from DNA damage - have been used to identify genotoxic substances (Tice *et al.*, 2000). A powerful approach for assessing DNA damage is the comet assay, assessing DNA strand breaks quantitatively and qualitatively (Singh *et al.*, 1988; McKelvey *et al.*, 1993; Fairbairn *et al.*, 1995). Since the comet assay was introduced (Ostling and Johanson, 1984), its broad application and the number of investigations using this technique have increased almost exponentially (Tice *et al.*, 2000), including genotoxicity risk assessment, biomonitoring, studies on DNA repair mechanisms and apoptosis research (Godard *et al.*, 1999).

Fish are particularly sensitive to pollutants that interact with DNA (Schnurstein and Braunbeck, 2001). In field studies high frequencies of neoplastic lesions in fishes from polluted areas has been reported (Murchelano and Wolke, 1991) emphasising the need for direct detection of DNA damage. For biomonitoring purposes, the comet assay was mainly carried out with adult fishes (Pandrangi *et al.*, 1995; Belpaeme *et al.*, 1998; Mitchelmore and Chipman, 1998a; Faverney *et al.*, 2001; Schnurstein and Braunbeck, 2001; Cestari *et al.*, 2004; Ferraro *et al.*, 2004), confirming that comet assay is a useful tool for assessment of genotoxic water pollution. According to the authors knowledge the available literature on the potential application on whole embryos are very few (Jarvis and Knowles, 2003; Kosmehl *et al.*, 2006). So, the present work aims to establish and evaluate an alkaline comet assay for the detection of DNA strand breakage in embryos of *Clarias gariepinus* and to monitor lead-caused DNA damage in these vulnerable stages after exposure to lead.

7.2 Results

Exposure to lead nitrate (100, 300 and 500 µg/l) caused DNA damage in terms of DNA strand breakage dependent on the concentration and the time of exposure

in embryos of *C. gariepinus*. First suitability of comet parameters (% TDNA, % HDNA, TL and TM, Fig. 32) were evaluated using the most sensitive one for the evaluation of lead-caused genotoxicity.

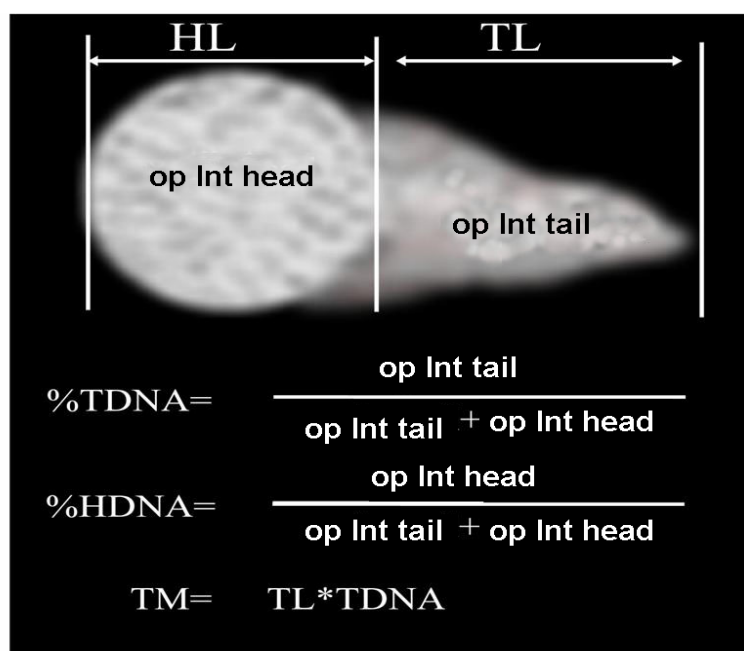


Fig. 32. Scheme of a comet designating the parameters used. HL= head length, TL= tail length, Op Int= optical intensity at 515-560 nm, % TDNA= the mean percentage of DNA in the tail, % HDNA= the mean percentage of DNA in the head, TM = tail moment.

7.2.1 Quantitative evaluation of the comet parameters (end points)

The percentage of the DNA comprising the tail (% TDNA), tail length (TL) and the tail moment (TM) were evaluated in the current work in order to identify the most sensitive parameter for the detection of the DNA strand breakage in embryonic stages of *C. gariepinus*. In the control all parameters analyzed for evaluation were constant over time (Table 9). Substantial variation, though not significant, was observed using the parameters TM and TL. TM fluctuated between 22.1 ± 4.1 in the 30h-PFS to 141.4 ± 29.4 in the 168h-PFS, revealing a 3-fold variation over time in the control group. The highest standard error (SE) was observed when using this parameter ($> 20\%$). TL revealed similar variation and SE, ranging from 2.1 ± 0.2 to 4.5 ± 0.5 (Table 9). % TDNA and % HDNA were highly comparable over time in the control (12.4 ± 1.4 to 15.9 ± 1.8 for % TDNA, 84.0 ± 1.8 to 87.6 ± 1.4 for % HDNA). Low SE was observed in both parameters (Table 9). In addition, using % TDNA or % HDNA, concentration-dependent DNA revealed a better resolution in the lead-exposed groups, suggesting a better potential for differentiation. TL and TM both revealed non-significant dose-

related responses (Table 9). Subsequently, % TDNA was used for the assessment of genotoxicity in embryos.

Table 9. DNA strand breakage after exposure to lead nitrate (control, 100, 300 and 500 µg/l) assessed by different parameters (TL, TM, % TDNA, % HDNA) at different developmental stages (30h-PFS, 48-PFS, 96h-PFS, 144h-PFS, 168h-PFS) in embryonic stages of *Clarias gariepinus*. Presented are mean ± SE (n=96 nuclei). Definitions of parameters see Figs. 32 & 34.

Age	Treatments µg/L	%TDNA	TL µm	TM	%HDNA
30h-PFS (prehatching stage**)	C	13.6±1.5 ^{a1}	2.1±0.2 ^{a1}	22.1±4.1 ^{a1}	86.4±1.5 ^{a1}
	100	26.2±1.5 ^{b1}	6.7±0.6 ^{b1}	223.7±29.5 ^{b1}	73.8±1.5 ^{b1}
	300	27.3±1.7 ^{b1}	8.2±0.7 ^{b1}	283.4±30.9 ^{b1}	72.7±1.7 ^{b1}
	500	31.5±1.6 ^{b1}	6.2±0.4 ^{b1}	243.5±23.9 ^{b1}	68.5±1.6 ^{b1}
48h-PFS	control	12.4±1.4 ^{a1}	2.1±0.2 ^{a1}	50.6±8.9 ^{a1}	87.6±1.4 ^{a1}
	100	26.6±1.5 ^{b1}	7.0±0.6 ^{b1}	231.6±29.8 ^{b1}	73.4±1.5 ^{b1}
	300	28.5±1.7 ^{b1}	8.6±0.7 ^{b1}	295.9±30.7 ^{b1}	71.5±1.7 ^{b1}
	500	31.7±1.6 ^{b1}	6.3±0.4 ^{b1}	245.4±23.9 ^{b1}	68.3±1.6 ^{b1}
96h-PFS	control	14.1±1.5 ^{a1}	3.1±0.3 ^{a1}	78.4±15.0 ^{a1}	85.9±1.5 ^{a1}
	100	22.8±1.6 ^{b1}	6.6±0.6 ^{a1}	209.7±30.0 ^{a1}	77.1±1.6 ^{b1}
	300	33.9±2.3 ^{c1}	18.0±3.3 ^{b2}	979.8±234.8 ^{b2}	66.1±2.2 ^{c1}
	500	41.6±2.2 ^{d2}	14.0±1.3 ^{b2}	699.0±83.1 ^{b2}	58.4±2.2 ^{d2}
144h-PFS	control	13.1±1.3 ^{a1}	2.8±0.3 ^{a1}	67.8±13.9 ^{a1}	86.9±1.3 ^{a1}
	100	22.0±1.5 ^{b1}	8.5±1.3 ^{a1}	256.0±42.0 ^{a1}	78.0±1.5 ^{b1}
	300	44.6±1.8 ^{c2}	25.8±2.7 ^{b2}	1344.7±155.2 ^{b2}	55.4±1.8 ^{c2}
	500	53.2±1.8 ^{d3}	26.4±1.9 ^{b3}	1459.6±110.3 ^{b3}	46.8±1.8 ^{d3}
168h-PFS	control	15.9±1.8 ^{a1}	4.5±0.5 ^{a2}	141.4±29.4 ^{a2}	84.0±1.8 ^{a1}
	100	22.9±2.3 ^{a1}	4.6±0.4 ^{a1}	171.7±23.5 ^{a1}	77.0±2.3 ^{a1}
	300	42.9±2.5 ^{b2}	25.4±3.8 ^{b2}	1318.8±242.6 ^{b2}	57.4±2.5 ^{b2}
	500	53.6±2.3 ^{c3}	26.3±2.0 ^{b3}	1592.3±158.4 ^{b3}	46.4±2.3 ^{c3}

^{a,b,c} level of significance analysed by non-parametric Bonferroni (n = 96 nuclei)

PFS = Post fertilization stage, % TDNA = Percentage of the DNA in the tail, % HDNA = percentage of the DNA in the head, TL = tail length, TM = tail moment.

** The hatching process started at 40 h after fertilization.

7.2.2 Genotoxicity in embryos after lead nitrate exposure

DNA damage quantified as % TDNA was constant in the control (Fig. 33a) and increased over time in the 300 µg/l and 500 µg/l treatments exposed to lead nitrate (Fig. 33c, d). Surprisingly, % TDNA in the 100 µg/l exposure did not increase over time, but was always slightly higher compared to the control (Fig. 33b). In all treatments, %

TDNA reached highest value after 144h-PFS and did not further increase. At a sampling point highest % TDNA though not significant until 96h-PFS were observed in the 500 $\mu\text{g/l}$ treatment (Table 9).

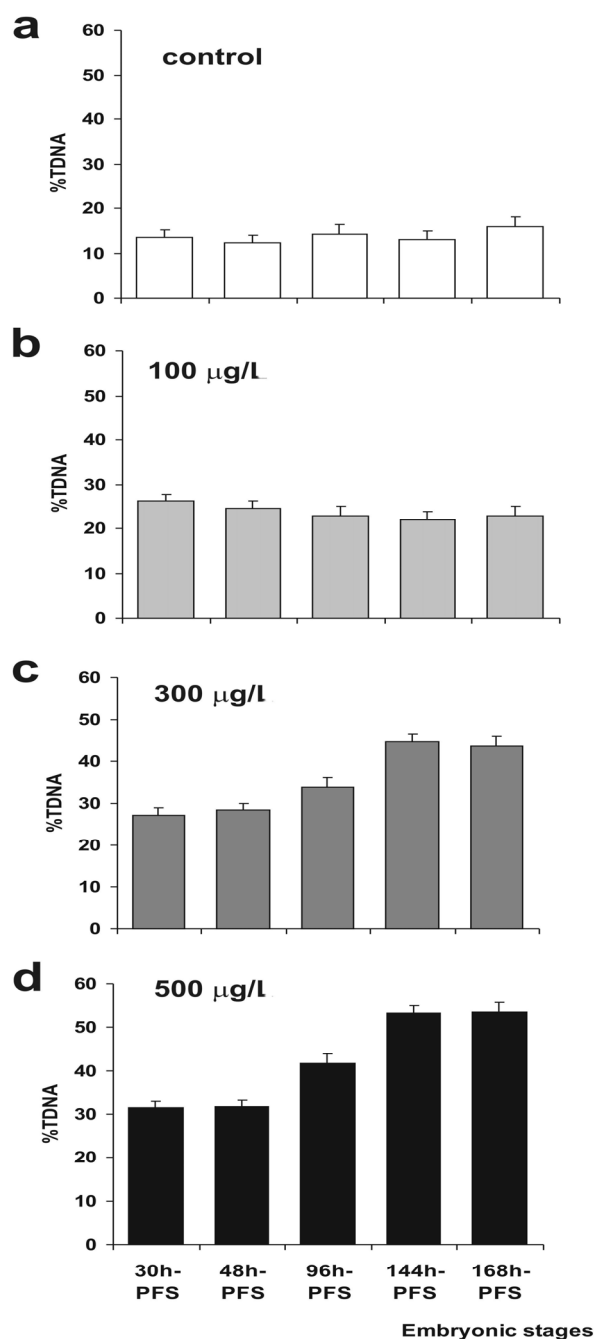


Fig 33. DNA strand breakage in embryos of *Clarias gariepinus* after lead nitrate exposure using % TDNA (means \pm SE, three replicates of 96 nuclei) of the control group (a) and after exposure to 100 $\mu\text{g/l}$ (b) 300 $\mu\text{g/l}$ (c) and 500 $\mu\text{g/l}$ (d) lead nitrate. % TDNA = the mean percentage of DNA in the tail, PFS = post fertilization stage.

The frequency of categories assessing the qualitative DNA damage (undamaged (% TDNA ≤ 10 %), low damaged (10 % $<$ % TDNA ≤ 25 %), median damaged (25 % $<$ % TDNA ≤ 50 %), highly damaged PFS (50 % $<$ % TDNA ≤ 75 %), and extremely damaged (75 % $<$ % TDNA) nuclei; Fig. 34) increased towards higher DNA damage categories with increasing dose of lead nitrate applied in comparison to the control (background level of DNA damage) in which the un-damaged nuclei have the highest frequency (51 % - 56.3 %) in all stages considered here (Fig. 35). Congruently, qualitative analysis revealed a shift in the frequency of DNA damage classes towards the higher damage ones, correlated both to time of exposure and lead concentration. Subsequently, at the end of the experiment nearly 60 % of the nuclei were highly or extremely damaged in the 300 and the 500 $\mu\text{g/l}$ treatment, compared to a low shift in the lowest concentration where high and extreme DNA damage was only observed in less than 15 % of the nuclei (Fig. 35d, e).

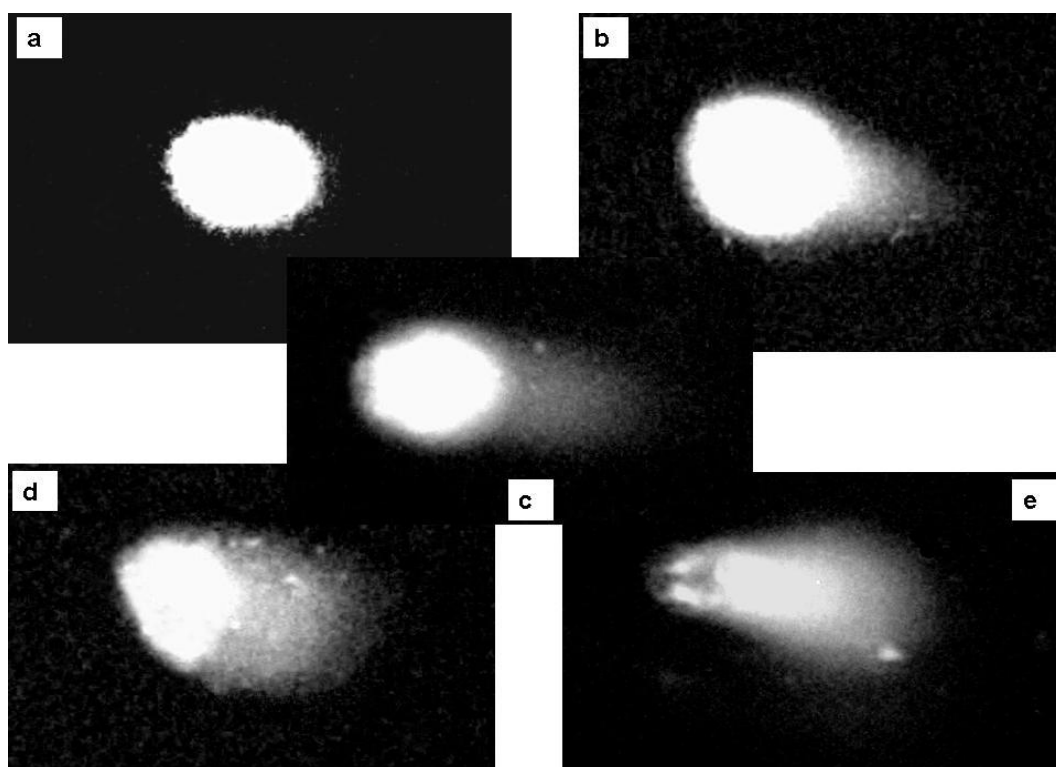


Fig. 34. Grade of DNA damage (representative stages) of embryos of *Clarias gariepinus*, exposed to lead nitrate. (a) undamaged nucleus of 96h-PFS (% TDNA ≤ 10 %), (b) low damage nucleus of 144h-PFS (10 % $<$ % TDNA ≤ 25 %), (c) median damaged nucleus of 96h-PFS (25 % $<$ % TDNA ≤ 50 %), (d) highly damage nucleus of 168h-PFS (50 % $<$ % TDNA ≤ 75 %), (e) extremely damage nucleus of 144h-PFS (75 % $<$ % TDNA)

No extremely damaged DNA was recorded in the pre-hatching stage (30h-PFS) and in the newly hatched embryos (48h-PFS) in all treated groups (Fig. 35a, b).

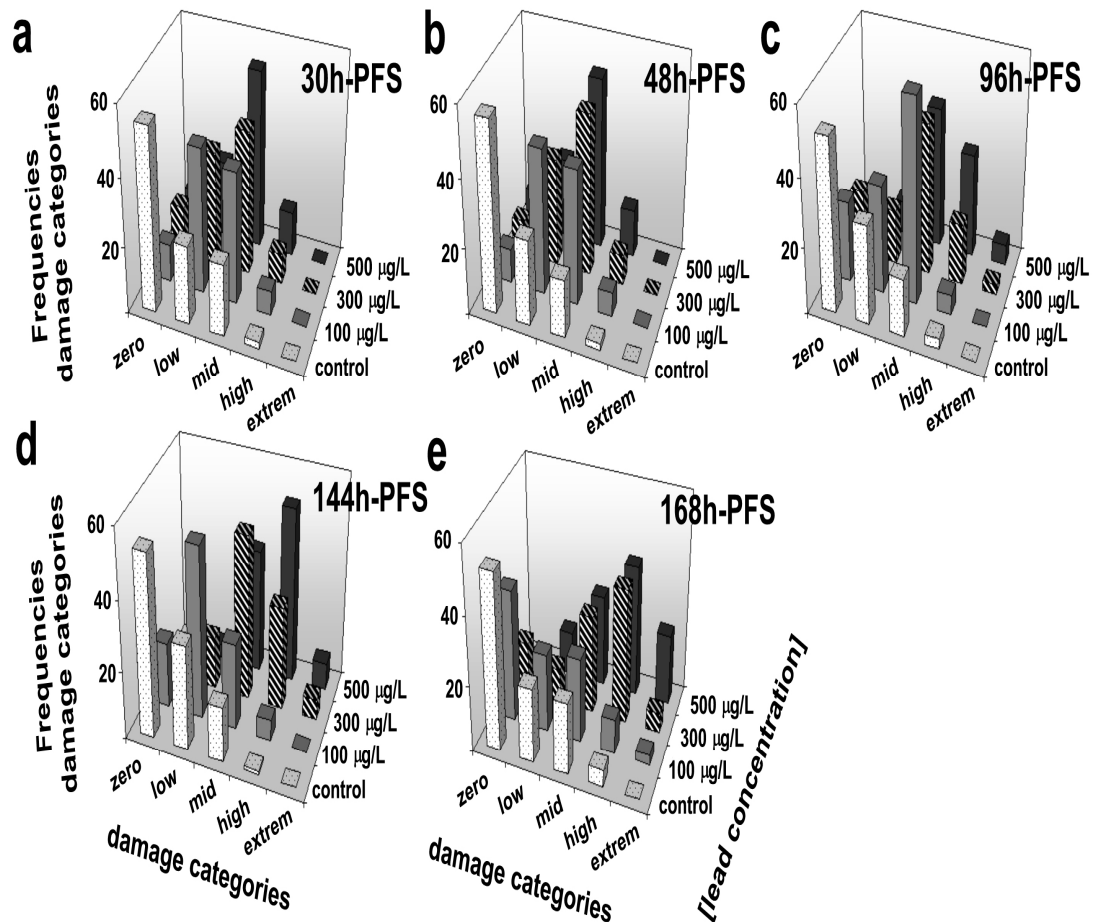


Fig. 35. Frequency (means \pm SE, 96 nuclei corresponding to 100 %) of DNA damage categories (zero, low, mid, high and extreme using % TDNA) in *Clarias gariepinus* embryos after the exposure to 0 (control), 100, 300, 500 $\mu\text{g/l}$ lead nitrate at (a) 30h-PFS, (b) 48h-PFS, (c) 96h-PFS, (d) 144h-PFS, (e) 168h-PFS. % TDNA = the mean percentage of DNA in the tail, PFS = Post fertilization stage.

Moreover, an impact index (II50) dose was calculated for each stage after grouping of the data into two categories; low-damage (included undamaged, low damaged and median damaged categories) and severely damaged (included highly damaged and extremely damaged categories). Comparable to the LC50, II50 represents the concentration where 50 % of the embryos were severely damaged. The II50 were 279.7, 264.8, 210.5, 165.4 and 165.7 $\mu\text{g/l}$ for 30h-PFS, 48h-PFS, 96h-PFS, 144h-PFS and 168h-PFS, respectively.

7.3 Discussion

We standardized the comet assay for whole embryos homogenate of *C. gariepinus* which; this method proved to be a valuable tool for the detection of genotoxic effects.

7.3.1 Quantitative evaluation of the comet parameters (end points)

Several parameters including % TDNA, TL and TM were used in the past to monitor DNA strand breakage by the comet assay techniques and these parameters were controversially discussed: % TDNA is the most frequently used (Anderson *et al.*, 1994; Mitchelmore and Chipman, 1998a; Pruski and Dixon, 2002; Hartmann *et al.*, 2003; Lee and Steinert, 2003; Kilemade *et al.*, 2004). Still, some authors (Olive *et al.*, 1990; Collins, 1992; Hellman *et al.*, 1995; Bauer *et al.*, 1998; Devaux *et al.*, 1998; Lee and Steinert, 2003) recommend tail length (TL) or tail moment (TM) for analysis. In particular, TM is believed to be an optimal parameter for the assessment of DNA breakage since both the absolute diversity of DNA breakage fragments (quantified as size-dependent migration by TL) and the relative amount of DNA breakage with regard to the initial DNA (considering optical intensity of DNA in the tail as % TDNA). Both parameters, TL and TM have been found inappropriate here due to the high variability in the control group throughout the experiment (high variation within the control group, high SE) and a reduced potential for differentiation of dose-dependent genotoxicity (levels of significance). Obviously, this is a result of the high variation in TL and a maximum detectable TL (at 26 μm , Table 9). We assume that the detectable size range of breakage fragments is reached fast and further increase of DNA damage does not cause further enlargement of size range. TM does not describe the genotoxic effect in a better way, probably overestimating the significance of the fragment size range by considering TL. The evaluation of commonly used parameters emphasize the need for standardization of the assay protocol in order to assure comparability, a claim previously requested by other authors (Schnurstein and Braunbeck, 2001). Our results clearly indicated that the % TDNA is the most appropriate criterion to quantify DNA strand breakage in fish embryos.

7.3.2 Genotoxicity in embryos after lead nitrate exposure

In the present work, embryonic stages revealed significant dose-related DNA damage response over time, since the % TDNA increased significantly with higher lead

concentrations. For the first time this is observed in early life stages of *C. gariepinus* congruent with studies in other species (Valverde *et al.*, 2001; Valverde *et al.*, 2002). Still, as organs of early life stages are not necessarily functioning, which in turn reduces capacity for bio-elimination, this is an important finding. In fish, a dose-dependent increase of % TDNA after lead nitrate exposure was reported in erythrocytes of *Hoplias malabaricus* (Ferraro *et al.*, 2004) and in a wide range of tissues in juvenile turbot *Scophthalmus maximus* (Kilemade *et al.*, 2004).

The mechanism of lead-caused toxicity is still discussed controversially. Recent evidence suggests that the genotoxic effects could be the result of several mechanisms, such as the induction of cellular immunity, oxidative stress, the inhibition of DNA metabolism and repair, and the formation of DNA and/or protein cross-link (Snow, 1992; Valverde *et al.*, 2001). DNA single breaks may be caused indirectly by the inhibition of DNA repair mechanism (reviewed by Lee and Steinert, 2003), the dysfunction of DNA polymerase or chromosomal aberration (Goyer and Moore, 1974). Thus, lead-caused genotoxicity may comprise DNA damage which cannot be assessed by monitoring DNA breakage. On the other hand, Lee and Steinert (2003) and Ferraro *et al.* (2004) concluded that single strand breakage is the main effect involved in lead-caused DNA damage, turning the comet assay in an ideal tool for the assessment of lead-caused genotoxicity.

After 24 hours of exposure (30h-PFS) % TDNA had increased in all treatment groups and after 42 hours (48h-PFS) in a dose-dependent way, being highest in the 500 µg/l concentration. Still, dose-dependence was not significant and significance was only revealed from 90 h on (96h-PFS to 168-PFS). After 144h-PFS (138 h exposure time), no remarkable genotoxic increase in % TDNA was observed in the 300 and 500 µg/l treatments. Similar results were previously reported by Kilemade *et al.* (2004) for the tissues of the juvenile turbot (*Scophthalmus maximus*), where the genotoxic response appeared stabile after 7 days. We conclude that DNA excision-repair processes were taking place after 138 h of exposure (144-PFS) preventing further DNA damage. Because DNA repair occurs by an enzymatic process, it may be subject to a threshold effect (Black *et al.*, 1996) requiring sufficient exposure time to be applied for induction or activation. Further studies will include the analysis of lead elimination and DNA repair mechanisms in fish embryos.

Extremely damaged nuclei (% TDNA >75 %) were not recorded in any of the pre-hatching stages, accompanied by low % TDNA. We conclude that low susceptibility in pre-hatching stages is most probably a consequence of the chorion which seems to protect the embryos from a range of external pollutants. Congruently, Osman *et al.* (2006) recorded that lead has a minor effect on the activity of some enzymes (G6PDH, LDH and PK) before hatching due to the presence of the hard chorion which protects the embryos. Mekawy and Osman (2006) postulated that the chorion started the hardening process three hours after fertilization which would provide protection from lead 6 hour after fertilization. This hypothesis was first suggested by Nguyen *et al.* (1999) with regard to heavy metal accumulation but is now further supported by the findings on reduced genotoxicity in pre-hatching stages. In addition, histopathological malformations confirmed a decreased sensitivity in pre-hatching stages. The same result has been recorded by Honkanen (2004) suggesting that the presence of an egg shell in pre-hatching stages seems to protect the embryos from a wide range of external stress factors during critical developmental events.

According to the definition of Yasuhara *et al.* (2003) a cell which contains more than 50 % of the total DNA in the tail comet is an apoptotic cell. Considering this definition, the highly and extremely damaged nuclei, in the present work could represent apoptotic nuclei. Consequently, lead could induce cell death by apoptosis in the embryos of the African catfish. Further studies are needed to determine the exact role and pathway of lead-caused apoptosis in embryos of fish.

Here the comet assay detected DNA strand breakage in the catfish embryos exposed to all three concentrations (100, 300 and 500 µg/l lead nitrate), turning the comet assay to be a more sensitive detection method than histopathology for low-level toxicity. Thus, it has been shown a useful tool for biomonitoring, urgently required in Egypt with regard to increasing environmental deposition of lead and bioaccumulation in human food recently observed (Abo-Gharbia and El-Sawi, 1999; Abou-Arab and Abou Donia, 2000).

SUMMARY

Fertilization and associated morphological and chemical variations of the egg surface and envelopes

Spermatozoon of *Clarias gariepinus* consisted of a head, midpiece and very long tail. The midpiece had an inverted conical shape forming an oval shaped structure with the head. The anatomy of spermatozoa seemed to be adjusted to the diameter of the inner aperture of the micropylar canal as contributing to the prevention of polyspermy. The ripe oocyte had four distinct layers: an outer most follicular layer (outer theca + inner granulosa layer), a median zona radiata (the chorion) and an inner oolemma or oocyte plasma membrane. The outer surface of the unfertilized is smooth whereas the fertilized egg acquired a network of projection 1 h post fertilization on the vegetal hemisphere. Different patterns of ornamentation on the egg surface were evident. These patterns were recorded in different regions and at different postfertilization times. The micropyle consisted of a funnel-shaped vestibule from the bottom of which a cylindrical micropylar canal extended. With developmental progress, the micropyle continued to narrowing with the formation of the micropylar disc. The development of such a micropylar disc made *C. gariepinus* eggs have a unique characteristic shape (a fur cap) that differ from that of other catfish.

The ovulated chorion consisted of one layer (zona radiata). Directly after fertilization, the single zona radiata was differentiated into zona radiata externa (ZRE) and zona radiata interna (ZRI). At ½ h and 1 h postfertilization stages, the chorion was differentiated into three layers, the double-layered coat, ZRE and ZRI. The ZRE disappeared completely in the micropyle, in the micropylar-like depression and in the vegetable pole. Four protein subunits were identified by SDS-PAGE in the chorion of the ovulated egg: three proteins of low molecular weights (MW) and one protein of relatively higher MW that had the highest percentages (29.8-47.89 %). Fertilization influenced the chorion proteins of the first three categories and changed them into proteins of higher molecular weight (108.93 to 129.7-133.6; 31.5-33.7 to 35.6-36.4; 25.3-26.9 to 28.3-29.2 KDa respectively).

Embryonic development

Entire embryogenesis was described in *C. gariepinus* (from fertilization to the end of exogenous feeding) with a focus on the organogenesis of the heart, notochord, eye, inner ear and digestive system. The first cleavages until the 64-cell stage (50 min - 4 h), the morula (4 h), the blastula (6 h) and gastrulation (12 h) were described. The pattern of cleavage recorded here for *C. gariepinus* did not markedly differ from that in some catfish, e.g. *Clarias batrachus*, *Pangasius sutchi* and *Mystus montanus* or most teleosts such as *Oryzias latipes*, *Danio rerio*, *Cichlasoma dimerus* and *Gadus morhua*. The beginning of gastrulation, defined by the onset of involution, resulted from the movement of embryonic cells over the surface of the yolk (epiboly) and was observed at the 50 % epiboly stage. Few hours before hatching the embryos showed twisting movements inside the egg. The hatching process started at 40 h post fertilization.

The newly hatched embryos were characterized by a large yolk sac which they nourished here for 120 h post-hatching (120h-PHS). The terminal mouth, the eyes and the operculum became well developed and the barbels resumed their normal shape 96h-PHS. The hatchlings possessed an undifferentiated digestive system, but 48h-PHS the digestive system was differentiated into a buccal cavity, oesophagus and intestine, although the mouth and the anus were still closed. At 96h-PHS, the connection between the oesophagus and the intestine was established and mouth and anus opened.

An unstricted notochord was observed until 120h-PHS. The notochord consisted of two types of cells (vacuolated cells and notochordal epithelium) enclosed in a fibrous sheath. The increase in vacuole size and the proliferation of vacuolated cells (with the course of development) directed pressure on the notochordal epithelium, transforming cuboidal to typical squamous cells.

In the eyes of the newly hatched embryo no pigment cells were recorded. Pigmentation was recorded shortly after hatching. At 24h-PHS, the neuroblastic layer was differentiated, but the cells of the different layers could not be distinguished morphologically. A central primordial lens was apparent. At 48h -PHS the crystalline lens differentiated and the retinal lamination was observed. Most of the retina layers could be recognized and the amount of melanin granules in the pigmented epithelium

increased further during this stage. At 96h-PHS, the deposition of the pigment granules in the pigmented epithelium and the retinal lamination increased further. The crystalline lens was a spherical structure consisting of two types of tissue. At 120h-PHS the pigmented epithelium was composed of a single layer of cubical cells and an outer nuclear layer comprising cones.

The inner ear of the newly hatched embryo was an oval-shaped vesicle with two otoliths and a sensory epithelium situated ventrally. The three semicircular canals (anterior vertical, horizontal and posterior vertical) of the inner ear with rudimentary cristae had formed at 48h-PHS. The sensory epithelium described here might enable the hatching embryos to maintain a 3-D orientation, detect vibration in the water and avoid moving pipet. Therefore, functionality of the inner ear can be assumed shortly after hatching in *C. gariepinus*.

Beating of the heart was observed as early as the 30 h post-fertilization. One day post-hatching few blood elements was recorded in the heart. Four defined compartments (bulbus arteriosus, ventricle, atrium, and sinus venosus) containing blood elements were visible at 96h-PHS.

Lead induced morphological and histological malformations during embryogenesis

Exposure to lead nitrate reduced the hatching rate from 75 % in the control group to 40 % in the group exposed to 500 µg/l lead. An overall delay in development was reported. In the control, hatching started 40 h after fertilization. In the groups exposed to 300 µg/l and 500 µg/l developmental time was prolonged and hatching started at 50 h post-fertilization. Four major categories of gross morphological abnormalities (irregular head shape, pericardial oedema, yolk sac oedema and notochordal defect) and two minor deformities (finfold defect and reduction of pigmentation) were observed. Some of the affected embryos were recorded with combinations of these. Three shapes of yolk sac oedema were observed in the present work comprising bag-shape oedema, balloon-shape oedema and oval-shape oedema. Different kinds of notochordal curvature were evident including lordosis, scoliosis, kyphosis and C-shaped body. The frequencies of the recorded morphological malformations increased significantly ($P < 0,05$) with increasing lead concentration in all

stages. Four histopathological categories comprising notochordal defect, gill malformation, eye malformation and detached skin were recorded. The degrees of histological lesions increased with increasing lead concentration and with length of exposure time. Unlike the morphological malformation, histopathological changes were only recorded in embryos exposed to highest dosages (300 µg/l, 500 µg/l lead nitrate). Embryos exposed to the highest two lead dosages showed arrested development of the gill comprising reduced development of cartilage and lamellae and the fusion of adjacent lamellae. Defected notochord was recorded in the embryos exposed to 300µg/l, 500µg/l lead nitrate. The eyes of the treatment groups exhibited different degrees of malformations including irregular and discontinuous pigmented epithelium, degeneration of retinal cells, and degeneration of the lens.

The lowest concentrations of lead were recorded in the control groups (0.3-1.0 µg/g wet wt). Accumulated lead increased significantly ($p < 0,05$) with increasing dose in all stages. Lead accumulated mainly in the chorion as suggested here by the highest concentration in the 30 h and 48 h post fertilization stage (48h-PFS).

Effects of lead nitrate on the activity of the metabolic enzymes G6PDH, LDH and PK

The activities of G6PDH, LDH and PK showed variability during the early embryonic stages (30h-168h PFS) under normal conditions. The pattern of the ontogenetic variation exhibited a significant ($p < 0.05$) decrease in the activities of all three enzymes till 96h-PFS. After 96h-PFS the activities of G6PDH and LDH increased toward the higher stages (144h-168h PFS). PK after 96h-PFS showed significant fluctuations in its activity.

The ontogenetic pattern of activity of the lead nitrate-treated embryos exhibited a pattern of enzyme activities similar to that of the normal embryos but with a significant variability in magnitude. However, within each embryonic stage, in comparison with the control, different patterns of enzyme activities were recorded. In 30h-PFS (pre-hatching stage) the activities of the three selected enzymes exhibited similar patterns. The activities increased significantly ($p < 0.05$) with 100 µg/l lead dose recording the highest value of activity during the whole experiment, and then the activities of the three enzymes decreased concomitantly with the higher doses. In the

subsequent post-hatching stages (48h–168h-PFS) the activity of G6PDH increased significantly ($p < 0.05$) from the control up to 500 $\mu\text{g/l}$ lead exposure. The activity of LDH was significantly inhibited ($p < 0.05$) with increasing concentration of lead nitrate during all post-hatched stages (48h–168 PFS) from the control to 500 $\mu\text{g/l}$ lead. The PK activity in each of the post-hatching stages (48h–168h-PFS) showed significant fluctuations (increase and decrease) with increasing lead doses.

Monitoring of DNA breakage after exposure to lead nitrate

For quantitative analysis, four commonly used parameters (tail % DNA - % TDNA, head % DNA - % HDNA, tail length – TL, tail moment - TM) were evaluated in the current work in order to identify the most sensitive parameter for the detection of the DNA strand breakage in embryonic stages of *C. gariepinus*. TL and TM have been found inappropriate here, due to high variability in the control group throughout the experiment (high variation within the control group, high standard error (SE)) and a reduced potential for differentiation of dose-dependent genotoxicity (levels of significance). % TDNA was highly comparable over time in the control. Low SE was observed. In addition, using % TDNA concentration-dependent DNA revealed a better resolution in the lead-exposed groups, suggesting a better potential for differentiation. Our results clearly indicated that, the % TDNA is the most appropriate criterion to quantify DNA strand breakage in fish embryos. Subsequently, % TDNA was used for the assessment of genotoxicity in embryos.

Embryonic stages revealed significant dose-related DNA damage response over time, since the % TDNA increased significantly with higher lead concentrations. After 24 hours of exposure (30h-PFS) % TDNA was increased in all treatment groups and after 42 hours (48h-PFS) in a dose-dependent way, being highest in the 500 $\mu\text{g/l}$ concentration. Still, dose-dependence was not significant and significance was only revealed from 90 h on (96h-PFS to 168-PFS). After 144h-PFS (138 h exposure times), no remarkable genotoxic increase in %TDNA was observed in the 300 $\mu\text{g/l}$ and 500 $\mu\text{g/l}$ treatment.

The frequency of categories assessing the qualitative DNA damage increased towards higher DNA damage categories in comparison to the control (background level of DNA damage) in which the un-damaged nuclei have the highest frequency (51 % -

56.3 %) in all stages considered here. Congruently, qualitative analysis revealed a shift in the frequency of DNA damage classes towards the higher damage ones, correlated to both time of exposure and lead concentration. Subsequently, at the end of the experiment nearly 60 % of the nuclei were highly or extremely damaged in 300 and 500 µg/l treatment, compared to a low shift in the lowest concentration where high and extreme DNA damage was only observed in less than 15 % of the nuclei. Extremely damaged nuclei (% TDNA >75 %) were not recorded in any of the pre-hatching stages, accompanied by low % TDNA. Moreover, the (IIC50) dose was calculated for each stage after grouping of the data into two categories; low-damage (included undamaged, low damaged and median damaged categories) and severely damaged (included highly damaged and extremely damaged categories). IIC50's were 279.7, 264.8, 210.5, 165.4 and 165.7 µg/l for 30h-PFS, 48h-PFS, 96h-PFS, 144h-PFS and 168h-PFS, respectively.

ZUSAMMENFASSUNG

Besamung und begleitende morphologische Veränderungen der Eioberfläche und Eihüllen

Spermatozoen bestehen aus einem Kopf, einem Mittelstück und einer sehr langen Geißel. Das Mittelstück hat eine konisch inverse Form und ergibt mit dem Kopf eine ovale Struktur. Die Anatomie des Spermatozoons ist offenbar an die innere Öffnung der Mikropyle entsprechend angepasst um Polyspermie zu verhindern.

Die reife Oocyte besitzt vier getrennte Schichten: Eine äußere follikuläre Schicht (äußere Theca und innere Granulosa-Schicht), eine mittlere Zona radiata (das Chorion) sowie eine innere Oolemma or Oocyten Plasma Membran. Die äußere Oberfläche des unbefruchteten Eies ist glatt wohingegen das besamte Ei eine Stunde nach der Besamung ein Netzwerk von Erhebungen am vegetativen Pol erkennen lässt. Weitere Muster von Oberflächenstrukturen waren auf der Eioberfläche an anderen Stellen und zu einem späteren Zeitpunkt nach der Besamung sichtbar. Die Mikropyle besteht aus einem tunnelartigen Vorhof aus dessen Grund sich ein zylindrischer Mikropylkanal erhebt. Mit fortschreitender Entwicklung und im Verlauf der Bildung der Mikropylenscheibe wurde der Mikropylkanal enger. Die Mikropylenscheibe hat ein pelzartiges Aussehen und unterscheidet sich von der anderer Weise.

Das Chorion des ovulierten Eies bestand aus einer einzigen Schicht (Zona radiata). Diese differenzierte sich direkt nach der Besamung in Zona radiata externa (ZRE) und Zona radiata interna (ZRI). Eine halbe bis eine Stunde nach der Besamung war das Chorion in drei Schichten untergliedert, einen doppelschichtigen Überzug sowie in ZRE und ZRI. Die ZRE erstreckte sich nicht bis in die Mikropyle und in die mikropylen-ähnlichen Eindellungen hinein und fand sich auch nicht am vegetativen Pol. Der Härtung des Chorions liegt eine Polymerisationsreaktion zugrunde in deren Verlauf unlösliche Eiweiße von höherem Molekulargewicht gebildet werden. Vier Proteinuntereinheiten wurden mit Hilfe der SDS-PAGE-Elektrophorese im Chorion ovulierter Eier gefunden: Drei Untereinheiten von geringerem und eins mit höherem Molekulargewicht (MW) welches mit der höchsten Häufigkeit auftrat (29.8-47.89 %). Besamung führte zu einer Erhöhung der MG der drei Proteinuntereinheiten mit den

niedrigen MG (von 108.93 nach 129.7-133.6; von 31.5-33.7 nach 35.6-36.4; von 25.3-26.9 nach 28.3-29.2 KDa).

Embryonalentwicklung

Der Verlauf der gesamten Embryogenese von *C. gariepinus* wurde beschrieben; von der Besamung bis zum Ende der exogenen Ernährung mit Betonung auf der Organogenese des Herzens, der Chorda, des Auges, des Labyrinths sowie des Verdauungssystems. 50 Minuten nach der Besamung begannen die ersten Zellteilungen, nach 4 Stunden war das Morula-Stadium erreicht, nach 6 Stunden das Blastula-Stadium und die Gastrulation nach 12 Stunden (entsprach dem 50 % Epibolie-Stadium). Das Muster der Zellteilungen, das bei *C. gariepinus* gefunden wurde, entsprach dem von anderen Welsen (*Clarias batrachus*, *Pangasius sutchi*, *Mystus montanus*) bzw. dem weiterer Teleosteer (*Oryzias latipes*, *Danio rerio*, *Cichlasoma dimerus*, *Gadus morhua*). Das Schlüpfen begann 40 Stunden nach der Besamung; einige Stunden vorher begann der Embryo mit Zuckbewegungen.

Der große Dottersack versorgte die frischgeschlüpften Embryonen für 120 Stunden (120h-PHS) mit endogener Nahrung. Das endständige Maul, die Augen und das Operkulum waren gut entwickelt 96h-PHS, zu dieser Zeit waren auch die Barteln normal gestaltet. Der Schlüpfling besaß ein undifferenziertes Verdauungssystem; 48h-PHS jedoch war das Verdauungssystem differenziert in Mundhöhle, Ösophagus und Mitteldarm, Mund und Anus waren noch geschlossen. 96h-PHS war die Verbindung zwischen Ösophagus und Mitteldarm etabliert und Mund und Anus waren offen.

Eine nicht eingeschnürte Chorda war vorhanden bis 120h-PHS. Die Chorda bestand aus zwei Zelltypen: Vakuolisierte Zellen und Chordaepithel, eingeschlossen in einer Bindegewebshülle. Im Verlauf der Entwicklung nahm die Größe der Vakuolen zu und auch die Zahl der vakuolisierten Zellen, gleichzeitig veränderten sich die Zellen des Chordaepithels von einem kubischen zu einem Plattenepithel.

In den Augen entwickelten sich die Pigmentzellen erst kurz nach dem Schlüpfen. 24h-PHS war die neuroblastische Schicht differenziert, jedoch konnten die Zellen der einzelnen Schichten nicht anatomisch unterschieden werden. Eine zentrale primordiale Linse war vorhanden. 48h-PHS war die kristalline Linse differenziert und

die retinale Schichtung vorhanden. 96h-PHS war die Zahl der Pigmentgranula im Pigmentepithel weiter erhöht und die retinale Schichtung weiter fortgeschritten. Die kristalline Linse war eine sphärische Struktur bestehend aus zwei Geweben. 120h-PHS bestand das Pigmentepithel aus einer einzigen Schicht kuboidaler Zellen und einer äußeren Zapfen-Schicht.

Das Labyrinth des Schlüpfings war ein oval geformtes Bläschen mit zwei Otolithen und einem ventral gelegenen Sinnesepithel. Die drei Bogengänge (vorderer vertikaler, horizontaler und hinterer vertikaler) mit rudimentären Cristae waren vorhanden 48h-PHS. Zu diesem Zeitpunkt scheint eine erste Funktionalität des Labyrinth gegeben.

Herzschlag begann etwa 30 Stunden nach der Besamung. Einen Tag nach dem Schlupf fanden sich erste Blutzellen im Herz. 96h-PHS war das Herz in vier Kompartimente untergliedert: Bulbus arteriosus, Ventrikel, Atrium und Sinus venosus.

Blei-induzierte morphologische und histologische Missbildungen im Verlauf der Embryogenese

Exposition mit Bleinitrat (500 µg/l) führte zu einer Reduktion der Schlüpftrate von 75 % (Kontrolle) auf 40 %. Eine generelle Verzögerung der Entwicklung wurde beobachtet. In der Kontrollgruppe begann der Schlüpfprozess 40 Stunden nach der Besamung. Bei den Gruppen mit der Exposition von 300 und 500 µg/l Bleinitrat war die Entwicklungszeit verlängert und das Schlüpfen begann 50 h nach der Besamung (PFS). Vier Kategorien größerer morphologischer Mißbildungen (irreguläre Kopfgestalt, Perikardialödem, Dottersacködem und Chorda-Defekte) und zwei geringere Formen von Mißbildung (Flossensaumdefekte und Reduktion der Pigmentierung) wurden beobachtet. Manche Embryonen besaßen Kombinationen dieser Mißbildungen. Drei Arten von Dottersacködem wurden beobachtet: Taschenartiges Ödem, ballonartiges Ödem und oval-geformtes Ödem. Verschiedenen Arten von Chordakrümmungen kamen vor wie z.B. Lordosis, Scoliosis, Kyphosis und C-geformter Körper. Die Häufigkeiten der Mißbildungen nahmen significant zu ($P < 0,05$) mit Zunahme der Bleinitrat-Konzentrationen in allen Stadien.

Vier histopathologische Kategorien wurden beobachtet: Chorda-Defekte, Kiemen- und Augenmißbildungen sowie Hautablösungen. Der Anteil der histopathologischen Defekte nahm mit steigenden Bleinitrat-Konzentrationen und mit der Dauer der Expositionszeit zu. Im Gegensatz zu den morphologischen Mißbildungen waren die histopathologischen Veränderungen nur bei den Embryonen, die den höheren Dosierungen (300 µg/l, 500 µg/l Bleinitrat) ausgesetzt waren, zu beobachten. Embryonen, die diesen Konzentrationen ausgesetzt waren, zeigten Wachstumsstop der Kiemen einschließlich reduzierter Entwicklung des Knorpels und der Kiemenlamellen und Fusionierung benachbarter Lamellen. Beschädigte Chorda fand sich bei Embryonen mit Expositionen von 300 und 500 µg/l Bleinitrat. Die Augen der Embryonen dieser Behandlungsserie zeigten verschiedene Stufen von Mißbildungen wie irreguläres und diskontinuierliches Pigmentepithel, Degeneration von Retinazellen sowie Linsendegeneration.

Bei den Untersuchungen zum Bleirückstand fanden sich die geringsten Konzentrationen von Blei in den Kontrollen (0.3-1.0 µg/g Naßgewicht). Mit zunehmender Expositionsdosierung stieg auch der Bleirückstand significant ($p < 0,05$) bei allen untersuchten Stadien an. Blei akkumulierte offenbar vorzugsweise im Chorion; dies lassen die höchsten gefundenen Konzentrationen in den Stadien 30 Stunden und 48 h nach Besamung (PFS) vermuten.

Effekte von Bleinitrat auf die Aktivitäten der Stoffwechselenzyme G6PDH, LDH und PK

Die Aktivitäten von G6PDH, LDH und PK zeigten Schwankungen während der frühen embryologischen Stadien (30h-168h PFS) in der Kontrollgruppe. Die Aktivitäten aller drei Enzyme nahmen zunächst significant ab ($p < 0.05$) bis zum 96h-PFS. Danach stiegen die Aktivitäten von G6PDH und LDH an hin zu den älteren Stadien (144h-168h PFS). PK hingegen zeigte nach 96h-PFS signifikante Schwankungen.

Die Embryonen, die Bleinitrat ausgesetzt waren, zeigten im Prinzip ein ähnliches Muster bezüglich der Schwankungen der Enzymaktivitäten wie dies schon bei den Kontrollembryonen beobachtet worden war, jedoch mit unterschiedlicher Größenordnung. Es wurden jedoch innerhalb von jedem Embryonalstadium, im Vergleich mit der Kontrolle, unterschiedliche Muster von Enzymaktivitäten beobachtet.

30h-PFS (vor dem Schlupf) zeigten die Aktivitäten der drei Enzyme ähnliche Werte. Die Aktivitäten stiegen significant an ($p < 0.05$) wobei bei der 100 µg/l Bleinitrat-Exposition die höchste Aktivität gemessen wurde; danach ergaben sich kontinuierlich niedrigere Werte in Abhängigkeit von den höheren Expositionen. In den folgenden Stadien nach dem Schlupf (48h–168h-PFS) nahm die Aktivität von G6PDH signifikant zu ($p < 0.05$) ausgehend von der Kontrollgruppe bis hin zur 500 µg/l Bleinitrat Exposition. Die Aktivität von LDH nahm significant ab ($p < 0.05$) mit zunehmender Konzentration von Bleinitrat während aller Entwicklungsstadien (48h-168 PFS) ausgehend von der Kontrolle bis hin zu der 500 µg/l Bleinitrat-Konzentration. Die PK Aktivität in jedem betrachteten Stadium nach dem Schlupf (48h–168h-PFS) zeigte signifikante Anstiege und Abfälle mit steigenden Bleinitrat-Konzentrationen.

DNA-Brüche nach Exposition mit Bleinitrat

Der Comet-Assay wurde für Homogenate von ganzen Embryonen standardisiert und zeigte sich im nachhinein als wertvoller Test zur Bestimmung von genotoxischen Effekten.

Zur quantitativen Analyse wurden vier üblicherweise genutzte Parameter bewertet (% DNA des Schwanzes = % TDNA, % DNA des Kopfes = % HDNA, Länge des Schwanzes = TL, Momentum des Schwanzes = TM) um den effektivsten Parameter für die Bewertung der DNA-Brüche bei den Embryonen herauszufinden. TL und TM erwiesen sich als ungeeignet bedingt durch die hohe Variabilität in der Kontrollgruppe (hoher Standardfehler) und das reduzierte Potential bezüglich der Differenzierung der dosisabhängigen Genotoxizität. % TDNA war sehr vergleichbar über die Zeit bei den Kontrollen. Eine geringer Standardfehler wurde beobachtet. Darüberhinaus erwies sich % TDNA als guter Indikator für DNA-Defekte in den Bleinitrat exponierten Embryonen. Unsere Ergebnisse zeigten eindeutig, daß % TDNA war das angemessenste Kriterium war, um DNA-Brüche bei Fischembryonen zu quantifizieren. Folglich wurde % TDNA benutzt zur Bewertung von Genotoxizität bei den Embryonen.

Die Embryonalstadien zeigten signifikante dosis- und zeitabhängige DNA-Schädigungen, da % TDNA significant erhöht war mit höheren Bleinitrate Konzentrationen. Nach 24 Stunden-Exposition (30h-PFS) war % TDNA erhöht in allen Behandlungsgruppen und nach 42 Stunden (48h-PFS) in einer dosisabhängigen Weise

wobei die höchsten Schädigungen bei Exposition von 500 µg/l Bleinitrat zu verzeichnen waren. Dennoch, die Dosisabhängigkeit war nicht signifikant und Signifikanz war nur vorhanden zwischen 96h-PFS und 168h-PFS. Nach 144h-PFS (138 h Expositionszeit) wurde kein Anstieg mehr in % TDNA gefunden in den Gruppen, die 300 µg/l und 500 µg/l Bleinitrat ausgesetzt worden waren.

Die Häufigkeit der Kategorien, welche für die Charakterisierung der qualitativen DNA-Schädigungen verwendet wurden, stiegen an in Richtung der höheren DNA-Schädigungskategorien verglichen mit der Kontrolle (Basiswerte), bei der die ungeschädigten Kerne die höchste Häufigkeit (51 % - 56.3 %) zeigten und zwar bei allen untersuchten Stadien. Gleichzeitig ergab die qualitative Analyse eine Verschiebung in der Häufigkeit der DNA-Schädigungsklassen in Richtung auf die höheren Schädigungsklassen hin in Abhängigkeit sowohl von der Expositionszeit als auch von der Bleinitratkonzentration. Entsprechend waren am Ende der Experimente 60 % aller Kerne stark bzw. sehr stark geschädigt bei den 300 und 500 µg/l Bleinitrat Expositionen, im Gegensatz zu einer geringeren Erhöhung bei der geringsten Konzentration (100 µg/l Bleinitrat), bei der starke und sehr starke Schädigungen nur bei weniger als 15 % der Kerne beobachtet wurden. Sehr stark geschädigte Kerne (% TDNA >75 %) fanden sich nicht bei den Embryonen vor dem Schlüpfvorgang, sondern nur geringe % TDNA-Werte. Darüberhinaus wurde die IIC50-Dosis für jedes Stadium kalkuliert, nachdem die Daten in zwei Kategorien untergliedert wurden: Gering geschädigt (nicht geschädigt, gering geschädigt, gering und mittelmäßig geschädigt) und stark geschädigt (stark und sehr stark geschädigt). Folgende IIC50-Werte ergaben sich: 279.7, 264.8, 210.5, 165.4 und 165.7 µg/l Bleinitrat für 30h-PFS, 48h-PFS, 96h-PFS, 144h-PFS und 168h-PFS entsprechend.

CONCLUSIONS

In order to study the embryo-toxic effects of lead nitrate, we first had to study the effect of fertilization on the hardening process of the chorion to clarify the role of the hardened chorion on the protection of the embryo from the pollutants. The chorion hardening is a process of initiated chain polymerization of substances within the membrane itself to form insoluble proteins of higher molecular weight. Fertilization influenced the chorion proteins and changed most of them into proteins of higher MW ones (108.93 to 129.7-133.6; 31.5-33.7 to 35.6-36.4; 25.3-26.9 to 28.3-29.2 KDa respectively). Also we had to study the embryonic development of *C. gariepinus* for providing us with a model for comparison when normal patterns of development are altered due the exposure to lead nitrate.

- Exposure of fertilized eggs to lead nitrate prolonged the incubation period and caused severe malformations. Delayed hatch may result from a retarded development or the inability of embryos to break the chorion. Physiologically, lead can also cause malfunctioning or even inhibition of egg shell digestive enzymes (chorionases).
- The lead exposure induced metabolic and osmotic disturbances; this subsequently could prevent the proper use of the energy stored in the yolk as suggested by the delayed hatch. Such disturbances often result in the hydration of the front portion of the yolk sac and lead subsequently to yolk sac oedema.
- Since the frequencies of the morphological malformations decreased with time, we conclude a lethal impact and selected mortality of abnormal embryos.
- Unlike the morphological malformation, histopathological changes were only recorded in embryos exposed to the highest dosages (300 µg/l, 500 µg/l lead nitrate). Thus, the morphological malformations were more sensitive for monitoring lead toxicity than the histopathological abnormalities.
- The significant increase of the activity of G6PDH in the post-hatching stages (48h – 168h- PFS) from the control up to 500 µg/l lead exposure represents protection against elevated levels of reactive oxygen species

(ROS) in cells exposed to an oxidant stress through the increased production of NADH.

- The inhibition in the activity of LDH with increasing concentrations of lead nitrate may be due to (i) ion imbalance or to (ii) intracellular action of metal subsequent to initial plasma membrane damage. This inhibitory effect may also be due to the direct binding of the lead to the enzyme and the formation of an enzyme-inhibitor complex.
- PK enzyme did not give a specific trend of response (increase or decrease) due to the exposure to the lead nitrate doses. Thus, the measurements of G6PDH and LDH but not PK activities could be useful biomarkers of lead pollution to reveal embryotoxic potential in fish.
- Several parameters including % TDNA, TL and TM were used in the past to monitor DNA strand breakage by the comet assay techniques and these parameters were controversially discussed. TL and TM have been found inappropriate here due to the high variability in the control group throughout the experiment and a reduced potential for differentiation of dose-dependent genotoxicity (levels of significance). Our results clearly indicated that the % TDNA is the most appropriate criterion to quantify DNA strand breakage in *C. gariepinus* embryos.
- Embryonic stages revealed significant dose-related DNA damage response over time, since the % TDNA increased significantly with higher lead concentrations till 144h-PFS. The mechanism of lead-caused toxicity could be the result of several mechanisms, such as the inhibition of DNA metabolism and repair, and the formation of DNA and/or protein cross-link. Single strand breakage is the main effect involved in lead-caused DNA damage, turning the comet assay in an ideal tool for the assessment of lead-caused genotoxicity.
- After 144h-PFS (138 h exposure times), no remarkable genotoxic increase in % TDNA was observed. We conclude that DNA excision-repair processes were taking place after 138 h of exposure (144h-PFS) preventing further DNA damage.

No specific response in the activities of all three enzymes was recorded in the pre-hatching stage (30h-PFS) after exposure to the lead nitrate doses. Also, no

extremely damaged nuclei (% TDNA >75 %) were recorded in the pre-hatching stage, accompanied by low % TDNA. This means the lead nitrate had a minute toxic effect on the prehatched embryos. We conclude that low susceptibility in pre-hatching stages is most probably a consequence of the chorion, which seems to protect the embryos from a range of external pollutants. The chorion of *C. gariepinus* started the hardening process 3 h after fertilization providing protection from lead 6 h after fertilization. This hypothesis was proved here by the highest concentration of lead in the 30h-PFS and 48h-PFS and is further supported by the findings on reduced genotoxicity in pre-hatching stages. In addition, histopathological malformations confirmed a decreased sensitivity in prehatching stages: The degree of malformations in the eyes, gills and notochord increased drastically after hatching. In our point of view, the protective capacity provided by a hardened chorion is a very interesting finding, well worth further studies. The selected biomarkers were sensitive detection methods for low-level toxicity of lead nitrate. Thus, these are useful tools for biomonitoring, urgently required in Egypt with regard to increasing environmental deposition of lead and bioaccumulation in human food recently observed.

LIST OF ABBREVIATIONS

% HDNA	Percentage of the damaged DNA in the head
% TDNA	Percentage of the damaged DNA in the tail
CPs	Carp pituitary suspension
DLC	Double Layered Coat
GSH	Glutathione
G6PDH	Glucose Dehydrogenase
LDH	Lactate Dehydrogenase
MLD	Micropyle-like Depression
MS-222	Tricaine methanesulfanate
MCH	Melanin-Concentrating Hormone
MSH	Melanocyte Stimulating Hormone
MW	Molecular weight
OE	Oveolated Egg
PAGE	Poly Acrylamide Gel Electrophoresis
PEP	Phosphoenol Pyruvate
PF	Post Fertilization
PFS	Post Fertilization Stage
PHS	Post Hatching Stages
PK	Pyruvate Kinase
PVS	Perivitelline space
PVSS	Secretions in PVS
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TL	Tail Length
TM	Tail Moment
ZR	Zona Radiata
ZRE	Zona Radiata Externa
ZRI	Zona Radiata Interna

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SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, die Dissertation selbständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben.

Ich habe mich nicht anderwärts als Doktorand beworben und besitze keinen entsprechenden Doktorgrad.

Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Landwirtschaftlich-Gärtnerischen Fakultät der Humboldt-Universität zu Berlin.

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